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PRINCIPAL INVESTIGATOR: Mark S. Smeltzer, PhD

CONTRACTING ORGANIZATION: University of Arkansas System
Little Rock, AR 72205

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Mark S. Smeltzer

E-Mail: smeltzermarks@uams.edu

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14. ABSTRACT. The goal of this project is to develop novel methods for the prevention and treatment of orthopaedic infections following traumatic injuries including those incurred on the battlefield. Using *Staphylococcus aureus* as a proof-of-principle pathogen based on its prominence as a cause of such infections and the severity of the infections its causes, we have undertaken studies to identify the most efficacious antibiotics and investigate the ability to conjugate these antibiotics to a novel bone targeting agent derived from a tetracycline moiety that binds hydroxyapatite. We have also demonstrated that the staphylococcal accessory regulator (*sarA*) plays a key role in the pathogenesis of orthopaedic infections owing in part to its impact on biofilm formation and intrinsic antibiotic resistance. Based on this, we have undertaken a search for small molecule inhibitors that could be used alone or in combination with conventional antibiotics and our bone targeting agent. Ultimately, these combinations will be evaluated based on their therapeutic efficacy in established animal models of traumatic bone infection.

15. SUBJECT TERMS
Staphylococcus aureus, biofilm, osteoblast, osteoclast, antibiotic, staphylococcal accessory regulator, *sarA*, SarA, bone targeting agent, daptomycin, ceftaroline, osteomyelitis

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INTRODUCTION

Infection is one of the most devastating complications following traumatic injury to the bone. These infections can be particularly problematic on the battlefield because the injury occurs under adverse conditions in which medical care must often be focused on controlling the life-threatening consequences of the injury without the time or resources required for intensive medical procedures to prevent infection pending transport to a safe, better equipped hospital. This same concern applies to many other forms of traumatic injury (e.g. car accidents) in which there is a time lapse between the incident and the availability of adequate medical care, the difference being that this critical time lapse is often much longer in the case of battlefield injuries. Thus, it is imperative to develop point-of-injury methods that can be used to minimize the possibility of infection, particularly bone infection, during this period. It is also imperative that the development of such methods take into account the fact that bone infections are remarkably recalcitrant to conventional antibiotic therapy. Thus, while the overall goal of this project is to optimize point-of-care therapy for the prevention of bone infection, an important secondary goal is to maximize the ability to treat these infections once they occur. We have chosen to focus our efforts on the bacterial pathogen *Staphylococcus aureus* based both on its predominance as a causal agent of such infections and the severity of the resulting infection; indeed, *S. aureus* bone infections often require extensive surgical debridement once the patient reaches a suitably equipped hospital environment. Moreover, even after such intensive medical and surgical intervention, the therapeutic outcome is often suboptimal and many times leads to repetitive debridement surgeries, loss of functional mobility, and even amputation. This comes at great financial and emotional cost and often leads to the loss of a highly trained and highly valued active duty service member. Our goal is to build on our years of experience studying *S. aureus* bone infection to develop novel, improved methods that can be used to treat an established infection and as a point-of-injury therapeutic to prevent the infection during the critical period between the injury itself and access to the extensive medical care necessary to more effectively deal with the injury and its potentially adverse consequences including infection.

KEYWORDS

Staphylococcus aureus, biofilm osteoblast, osteoclast, antibiotic, staphylococcal accessory regulator, bone targeting agent, daptomycin, oxacillin, ceftaroline, osteomyelitis

MAJOR GOALS/SPECIFIC AIMS

The major goals of this project are reflected in the specific aims of our Peer Reviewed Orthopaedic Research Program (PRORP) Expansion Award (Award #W81XWH-15-1-0716, Log #OR140356) as detailed below (modified from original application to reflect experimental progress in each aim):

Aim 1: Evaluate existing antibiotics and their bone-targeting (BT2-minipeg-2)-conjugates in the context of a biofilm. Neale et al. (2009) described the use of a hydroxyapatite-binding moiety (BT2-minipeg-2) derived from tetracycline as a targeting agent for the enhanced delivery of estradiol to the bone. In a subsequent study, Karau et al. (2013) examined the efficacy of this approach as a means of delivering vancomycin to enhance therapeutic efficacy. The enhanced delivery of vancomycin to bone was confirmed in these studies, as was an enhanced therapeutic effect in the specific context of bone infection. However, this had the adverse consequence of causing significant pathology in the kidney. In a more detailed pharmacokinetic analysis, we confirmed the bone targeting properties of BT2-minipeg-2 conjugated vancomycin, but also confirmed that this was associated with increased levels of vancomycin in the plasma (Albayati et al., 2015), an observation that likely accounts for the increased nephrotoxicity observed in these earlier studies (Karau et al., 2013). More recently, we demonstrated that BT2-minipeg-2 in and of itself does not have toxic properties (Albayati et al., BT2-minipeg-2 is a safe carrier for the targeted systemic delivery of antibiotics in the treatment of bone infections, AAPS (American Association of Pharmaceutical Sciences) Journal, see appendix), thus confirming that it was likely that the vancomycin component of the BT2-minipeg-2 conjugated vancomycin conjugate that was responsible for the observed toxicity. Taken together, these results support the use of the BT2-minipeg-2 as a bone targeting agent when delivered systemically, but they also suggest that vancomycin is not an appropriate antibiotic in that

regard owing to its nephrotoxicity. Thus, one of our first goals was to evaluate and prioritize alternative antibiotics for conjugation to BT2-minipeg-2 based on their relative activity in the context of an established biofilm. Having accomplished this task (Meeker et al., 2016), we have now turned our attention to optimizing methods to achieve conjugation of BT2-minipeg-2 to those antibiotics that exhibited the greatest activity in this context. Most notable among these were daptomycin and ceftaroline. To date, we have generated an active BT2-minipeg-2 conjugate to daptomycin, thus putting us in a position to pursue *in vivo* studies assessing toxicity and therapeutic efficacy. We have encountered greater difficulty in creating the ceftaroline conjugate, but we have made progress in that regard (see below). To increase therapeutic options, we also generated a BT2-minipeg-2 conjugate to ciprofloxacin, and for the reasons detailed below we are continuing to work on generating a conjugate to a novel linear lipopeptide antibiotic derived from paenipeptin (Huang et al, 2017).

Aim 2: Identify small molecule inhibitors of *S. aureus* biofilm formation and the pathogenesis and therapeutic recalcitrance of orthopaedic infections. We have demonstrated that mutation of the staphylococcal accessory regulator (*sarA*) limits the ability of *S. aureus* to form a biofilm to a degree that can be correlated with increased antibiotic susceptibility (Atwood et al., 2016, Weiss et al., 2009) and that it also limits virulence in animal models of *S. aureus* infection including bacteremia (Rom et al., 2017, Zielinska et al., 2012) and acute, post-traumatic osteomyelitis (Loughran et al., 2016). We have also confirmed that mutation of *sarA* limits biofilm formation to a greater extent than mutation of any other *S. aureus* regulatory locus we have examined and that, in those cases in which a mutation results in an enhanced capacity to form a biofilm, concomitant mutation of *sarA* reverses this effect (Atwood et al., 2015). Thus, we believe that small molecule inhibitors of *sarA*-mediated regulation could be used to great therapeutic advantage in the context of all diverse forms of *S. aureus* infection including biofilm-associated bone infections that arise following traumatic injury. This belief accounts for the experimental focus in our previous PRORP proposal (OR090571: *sarA* as a target for the prevention and treatment of staphylococcal biofilm associated infection). Based on this, the focus in this aim is to identify such inhibitors, verify their biological activity, and assess the ability to conjugate these inhibitors to our BT2-minipeg-2 bone targeting agent as a means of enhancing their delivery to bone and thereby limiting the extensive bone destruction and remodeling that characterizes acute, post-traumatic bone infection. This is being done using a high-throughput screen of an existing small molecule library as well as more targeted screens of analogues of promising compounds identified either in our primary screen or based on reports from the literature. We have also begun to employ molecular modeling, which allows us to make predictions regarding the likelihood that a given compound will be an effective inhibitor of SarA function and to at least begin to obtain mechanistic information regarding the means by which it functions (see below).

Aim 3: Evaluate the therapeutic efficacy of the agents identified and optimized in Aims 1 and 2 in the context of bone infection. Our overall goal is to develop novel methods that can be used for the prevention and treatment of bone infection following traumatic injury. This will require the use of animal models that accurately reflect the clinical problems that define these infections. In this aim, we will determine the extent to which the antibiotics we identify in Aim 1, and the *sarA* inhibitors we develop in Aim 2, can be used to prophylactic and/or therapeutic advantage by defining their efficacy, both alone and in combination with each other, in validated animal models that accurately reflect the clinical problem of bone infection (Loughran et al., 2016). As noted above, and described in detail below, we have demonstrated that BT2-minipeg-2 is not toxic, and we have generated BT2-minipeg-2 conjugates to ciprofloxacin and daptomycin. Thus, we are now in a position to test both the pharmacological properties and therapeutic efficacy of these alternative antibiotic formulations.

The ability to accomplish these aims can be summarized in the 9 tasks listed below. We have made significant progress in this regard, but as would be expected given the necessity of completing certain tasks before others can be undertaken, most of our work to date has focused on the first 5 of these tasks. However, we have confirmed that our BT2-minipeg-2 bone targeting agent is not toxic in and of itself (Albayati et al., manuscript submitted, see appendix), and given that we have generated BT2-minipeg-2 conjugates to promising antibiotics (e.g. daptomycin, Meeker et al., 2016), we have now begun to

undertake *in vivo* studies to evaluate the toxicity and therapeutic efficacy of alternative BT2-minipeg-2 antibiotic conjugates in the specific context of bone infection (Task 9).

TASK LIST

Task 1: Compare antibiotics active against MRSA in the context of a biofilm (completed).

Task 2: Determine whether the most promising antibiotics can be conjugated to BT2-minipeg-2 without compromising efficacy in the context of a biofilm (completed).

Task 3: Evaluate *in vivo* pharmacological properties of BT2-minipeg-2 antibiotic conjugates in the context of bone targeting (in progress).

Task 4: Optimize previously identified compounds based on inhibition of *sarA*-mediated regulation (in progress).

Task 5: Screen additional compounds to identify small molecule inhibitors of *sarA*-mediated regulation (in progress).

Task 6: Expand the screen of small molecule inhibitors to include additional staphylococcal strains and species (in progress).

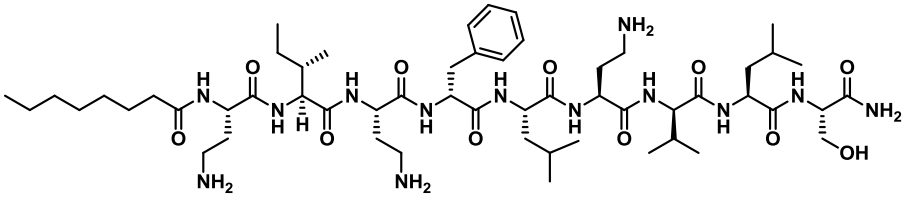
Task 7: Evaluate conjugation of the most promising *sarA* inhibitor to BT2-minipeg-2 (pending).

Task 8: Evaluate *in vivo* pharmacological properties of the most promising *sarA* inhibitor and its BT2-minipeg-2 conjugate (pending).

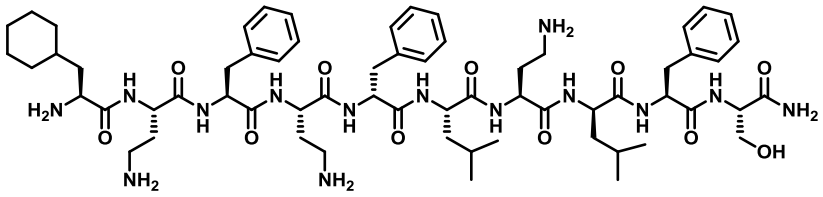
Task 9: Evaluate the efficacy of the most promising small molecule inhibitor and the most promising antibiotic *in vivo* with and without conjugation to BT2-minipeg-2 (in progress).

ACCOMPLISHMENTS

Task 1: Compare antibiotics active against MRSA under standard testing conditions in the context of an established biofilm. We compared the therapeutic efficacy of daptomycin, ceftaroline, vancomycin, telavancin, oritavancin, dalbavancin and tigecycline in the specific context of an established biofilm under both *in vitro* and *in vivo* conditions. These antibiotics were chosen because all are effective against methicillin-resistant *S. aureus* (MRSA). However, to limit the use of these antibiotics to patients infected with MRSA, we also included oxacillin in these studies. The results confirmed that daptomycin and ceftaroline have significantly greater efficacy than any other antibiotic tested, including what is widely considered the “last resort” antibiotic vancomycin (Meeker et al., 2016). Thus, much of our effort moving forward into Task 2 has focused on these two antibiotics. However, we have continued to explore the relative efficacy of additional antibiotics including ciprofloxacin and one promising antibiotic that remains under development. Specifically, Dr. En Huang in the College of Public Health at the University of Arkansas for Medical Sciences (UAMS) has identified a novel linear lipopeptide antibiotic derived from paenipeptin (Huang et al, 2017). He also developed a synthetic scheme for this antibiotic (paenipeptin C’, see below), which greatly facilitates the ability to obtain this antibiotic in large amounts. In addition, Dr. Huang has generated a number of analogues of this lipopeptide. One of these (analogue **17**) has a minimum inhibitory concentration (MIC) ranging from 0.5 to 8.0 µg per ml across a wide spectrum of bacterial pathogens including *Acinetobacter baumannii* (ATCC 19606), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecium* (ATCC 19434), and two different strains of *S. aureus* (ATCC 29213 and ATCC 43300), with the general trend being greater activity against Gram-negative pathogens by comparison to Gram-positive species.



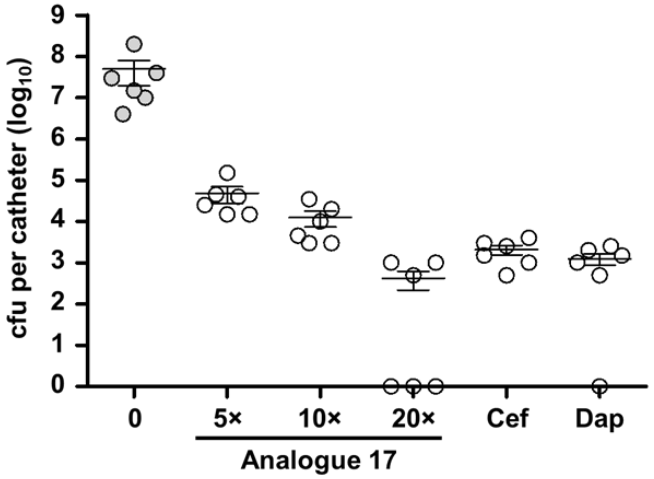
Chemical structure of paenipeptin C'



17

Chemical structure of paenipeptin analogue **17**

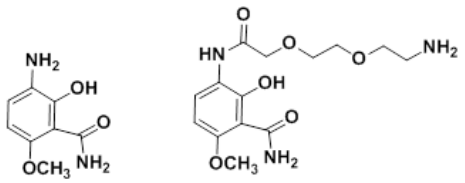
Perhaps more importantly, we demonstrated that, even when used at a concentration only 5 times (5x) the MIC for *S. aureus* as defined under standard planktonic conditions, analogue **17** exhibits significant antibacterial efficacy in the context of an established biofilm formed by the MRSA strain LAC. Moreover, when tested at a concentration corresponding to 20x the MIC for each antibiotic, the activity of analogue **17** in the context of an established biofilm was found to be comparable, if not greater than, that of ceftaroline (Cef) and daptomycin (Dap). A manuscript fully describing these results, the methods used to generate these analogues, and the details of their activity against different bacterial species is currently under review (Moon et al., Structure-activity relationship studies of linear lipopeptide paenipeptins with potential for eradicating biofilms and sensitizing Gram-negative bacteria to rifampicin, submitted to Journal of Medicinal Chemistry, see appendix). Based on these studies, efforts are currently underway to conjugate analogue **17** to BT2-minipeg-2 (Task 2).



Activity of paenipeptin C' analogue **17** in an established *S. aureus* biofilm. Relative activity was assessed using our established catheter assay (Meeker et al. 2016). Results are shown as colony-forming units (cfu) per catheter remaining after 3 days in the absence of antibiotic exposure (0) or after exposure to the indicated antibiotics at the indicated concentrations. Analogue **17** was assessed at concentrations of 5, 10 and 20x the MIC for the *S. aureus* strain under study. Based on our previous experiments (Meeker et al., 2016), ceftaroline (Cef) and daptomycin (Dap) were tested at 20x the MIC for each antibiotic as defined using the same test strain.

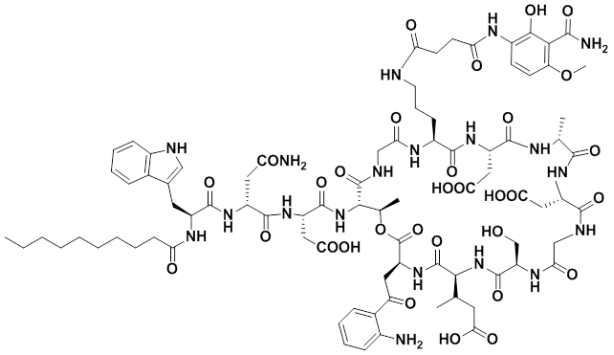
Task 2: Determine whether the most promising antibiotics can be conjugated to BT2-minipeg-2 without compromising efficacy in the context of a biofilm. Based on the results of Task 1, we have undertaken efforts to conjugate the most efficacious antibiotics, including analogue **17** (Task 1), to our bone-targeting agent. This has proven challenging from both a chemical and biological point of view.

Specifically, we compared conjugates of various antibiotics to both BT2 and BT2-minipeg-2, with the latter including a water soluble linker that separates the BT2 agent from the antimicrobial agent:

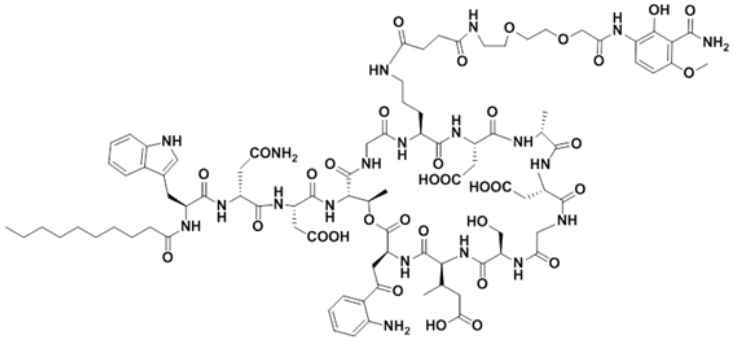


BT2 BT2-minipeg-2

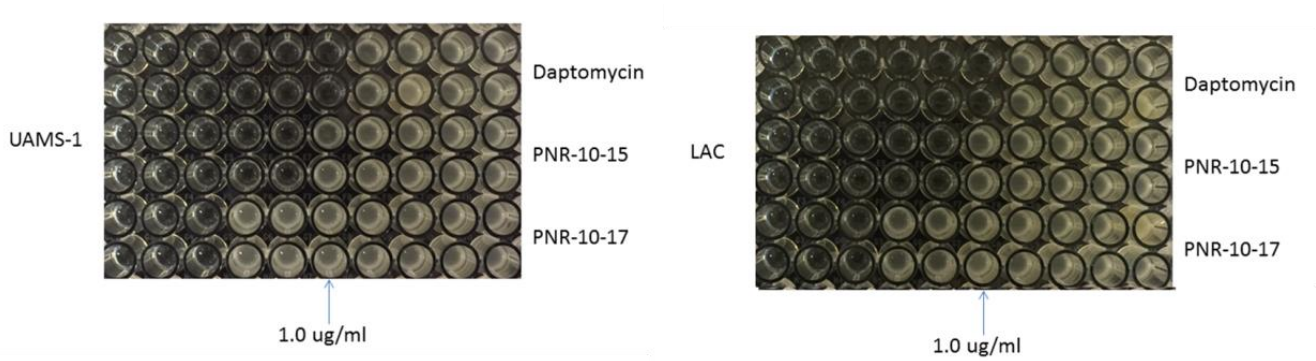
As assessed based on standard microtiter plate MIC assays, we confirmed that neither BT2 nor its BT2-minipeg-2 derivative have antimicrobial activity in and of themselves (see previous progress report). This absence of antibacterial activity is not necessarily desirable, as antibacterial activity of the targeting agent itself could prove useful. However, it does suggest that these agents are less likely to be toxic, which is an issue that we have experimentally addressed in detail as described below. We also generated BT2 and BT2-minipeg-2 conjugates with daptomycin (**PNR-10-17** and **PNR-10-15**, respectively) and tested their impact on antibiotic efficacy. In the case of BT2 itself (**PNR-10-17**), when tested at equal molarity the MIC was increased ~8-fold, while in the case of BT2-minipeg-2 (**PNR-10-15**) it was increased ~2-fold. This suggests that BT2-minipeg-2 may be the preferred targeting agent by comparison to BT2 itself, although it remains to be determined whether this is a generalizable observation or one that is specific for daptomycin. Nevertheless, in our studies examining a BT2-minipeg-2 vancomycin conjugate, uptake in the bone was increased almost 50-fold by comparison to vancomycin alone (Albayati et al. 2015). Thus, we do not believe an increase in MIC of 2-fold diminishes the potential of the BT2-minipeg-2 daptomycin conjugate as a therapeutic agent. We also confirmed that the activity of the BT2-minipeg-2 daptomycin conjugate was evident with both the USA200 methicillin sensitive strain (MSSA) UAMS1 and the USA300 MRSA strain LAC (Task 6).



PNR-10-17: Daptomycin BT2-succinic acid conjugate

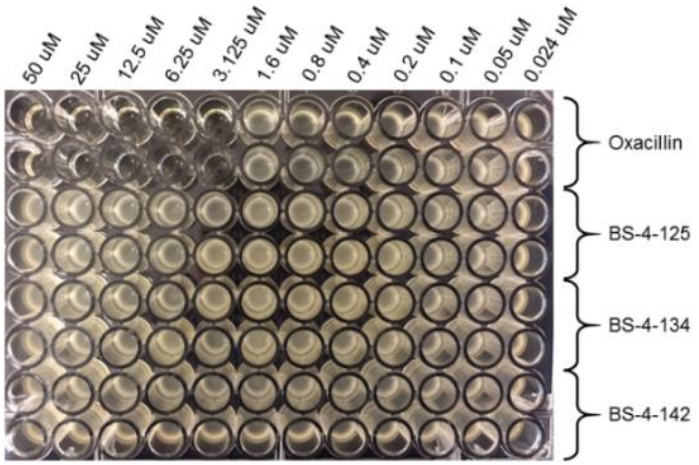


PNR-10-15: Daptomycin BT2-minipeg-2 conjugate



Note: The arrow indicates the CLSI-defined breakpoint MIC for a daptomycin sensitive strain of *S. aureus.* Each BT2 conjugate was tested at the molar equivalent of the concentration of daptomycin itself, which was included as a control in each assay. **PNR-10-15** is the BT2-minipeg-2 daptomycin conjugate, while **PNR-10-17** is the conjugate to BT2 itself.

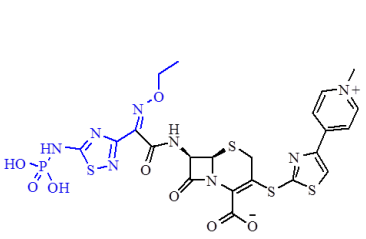
We also generated several variations of oxacillin conjugated to BT2 **(BS-4-125**) or BT2-minipeg-2, with the latter including two variants based on the length of the minipeg linker (**BS-4-134** and **BS-4-142**). However, unlike our daptomycin conjugates, antibacterial activity was essentially abolished with all of these conjugates as illustrated below (unlike the daptomycin assays detailed above, these studies were limited to the methicillin-sensitive *S. aureus* (MSSA) strain UAMS-1 because oxacillin is not active against MRSA). Given our success with other antibiotics, most notably daptomycin, and the fact that these other antibiotics are active against both MSSA and MRSA, we have abandoned, at least temporarily, our efforts to generate an active BT2-minipeg-2 oxacillin conjugate in favor of focusing on these other antibiotics.



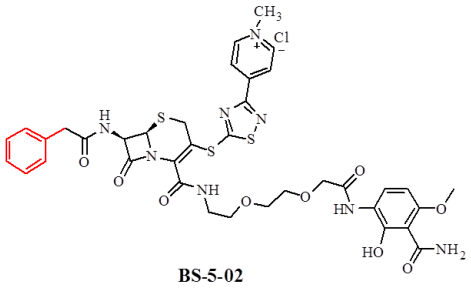
Lack of antimicrobial activity of BT2 (BS-4-125) and BT2-minipeg-2 conjugates (BS-4-134 and BS-4-142) to oxacillin. All three conjugates tested at molar equivalents exhibited a ≥32-fold increase in MIC relative to oxacillin itself.

We have also demonstrated that the 5th generation cephalosporin ceftaroline, which is unique among beta lactam antibiotics in that it is active against methillin-resistant *S. aureus* (MRSA), exhibits activity in the context of an established biofilm that is comparable to that of daptomycin (Meeker et al., 2016). However, generating an active BT2-minipeg-2 ceftaroline conjugate has proven particularly problematic, owing in part to the fact that it is commercially available only as a prodrug (ceftaroline fosamil) that is not easily conjugated to our bone targeting agent(s). Given its activity against MRSA, and the favorable safety profile of beta lactams in general, we have made a considerable effort to overcome this problem. To this end, we synthesized ceftaroline analogues **(BS-5-02** and **BS-5-53**), one of which has already been successfully conjugated to BT2-minipeg-2 (**BS-5-02**). In addition, Yarlagadda et al. (2016) recently described a dipicolyl-vancomycin conjugate that contains a zinc-binding moiety and is active even against vancomycin-resistant *Enterococci* (VRE). Based on this, we also synthesized a dipicolylaminobutane-ceftaroline zinc-containing compound (**BS-6-19**) and a BT2-minipeg-2 conjugated dipicolyl amine analogue (**BS-6-59**) that can be used to generate a similar Zn complex with ceftaroline. Finally, using a novel method developed in our

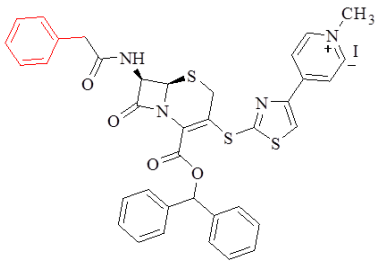
laboratory, we also successfully synthesized a BT2-minipeg-2 conjugate to ceftaroline fosamil (**BS-6-71*)***. Spectral analysis and efficacy testing of all of these compounds is pending.



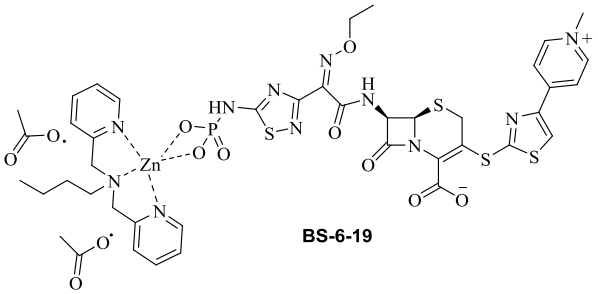
Ceftaroline fosamil



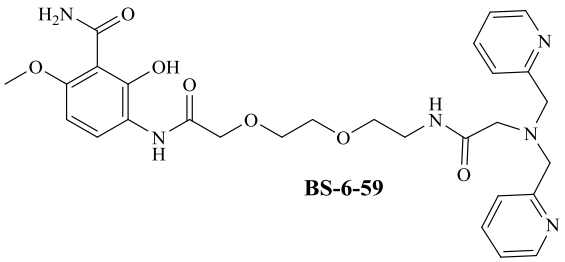
BS-5-02



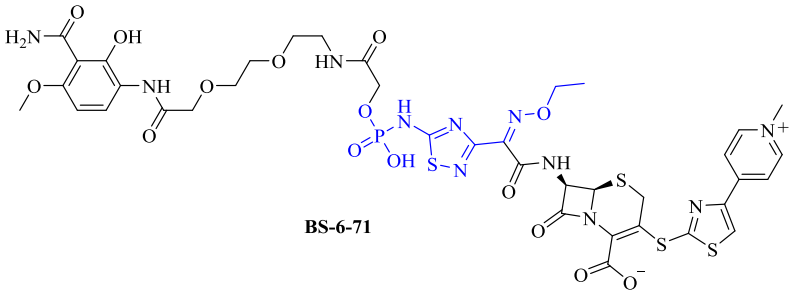
BS-5-53



BS-6-19

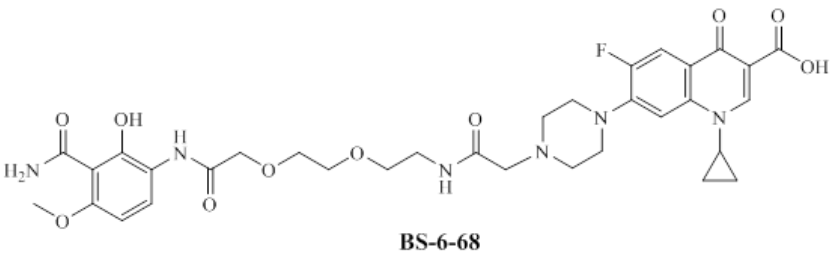


BS-6-59



BS-6-71

Finally, as noted above, we have begun to investigate the ability to conjugate BT2-minipeg-2 to additional antibiotics including ciprofloxacin and the novel linear lipopeptide paenipeptin analogue **17**. Studies investigating the conjugation of analogue **17** are ongoing, but the BT2-minipeg-2 ciprofloxacin conjugate was successfully generated without an appreciable loss of antibacterial activity. Specifically, using the *S. aureus* strain UAMS-1, the MIC for the BT2-minipeg-2 conjugate of ciprofloxacin was only 4-fold higher than that observed for ciprofloxacin itself (data not shown). As discussed above in the context of our BT2-minipeg-2 daptomycin conjugate, given the ~50-fold increase in bone targeting, we do not believe this decrease in activity precludes further study of our BT2-minipeg-2 ciprofloxacin conjugate *in vivo*.



BS-6-68

Chemical structure of BT2-minipeg-2 ciprofloxacin.

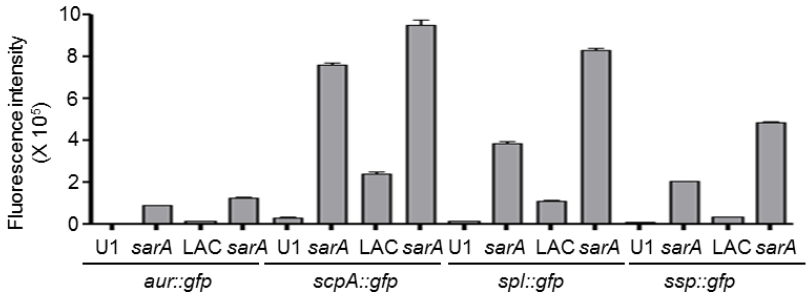
In contrast, we also evaluated BT2-minipeg-2 conjugates to moxifloxacin and sparfloxacin, and in both cases conjugation resulted in an increase in the MIC for UAMS-1 of ⩾32-fold. Thus, these conjugates have been excluded from further consideration.

Task 3: Evaluate *in vivo* pharmacological properties of BT2-minipeg-2 antibiotic conjugates in the context of bone targeting. The primary focus of this task is to evaluate BT2-minipeg-2 conjugates to antibiotics prioritized in Task 1 and shown to retain their activity after conjugation as assessed in Task 2. While we have encountered difficulties with certain antibiotics as detailed above, we have also had success with others, most notably ciprofloxacin and daptomycin. Thus, we are now in a position to move forward with studies evaluating the *in vivo* pharmacological properties of these BT2-minipeg-2 antibiotic conjugates (Task 9). However, as noted above, Karau et al. (2013) found that the dosing regimen required to achieve an enhanced therapeutic effect with BT2-minipeg-2 vancomycin was associated with significant nephrotoxicity, thus precluding its prophylactic or therapeutic use at least via systemic routes of administration. Based on this, we first wanted to verify that the nephrotoxicity observed with the BT2-minipeg-2 vancomycin conjugate was a function of altered pharmacokinetic (PK) properties of vancomycin and not a function of BT2-minipeg-2 itself. Thus, we undertook pharmacological studies to assess the targeting efficacy, pharmacokinetic (PK) properties, and toxicity of BT2-minipeg-2.

To this end, we undertook studies in which BT2-minipeg-2 was administered by intraperitoneal (IP) injection using an intensive dosing regimen of twice daily for 21 days. This dosing regimen was chosen because it was the same regimen in which the BT2-minipeg-2 vancomycin conjugate was shown to exhibit enhanced therapeutic efficacy in a rat osteomyelitis model but also to exhibit severe nephrotoxicity (Karau et al., 2013). The results of these studies, which will be presented at the annual meeting of the American Association of Pharmaceutical Sciences (AAPS) in San Diego, CA in November, 2017 and have been accepted for publication in the AAPS Journal (Albayati et al., BT2-minipeg-2 is a safe carrier for the targeted systemic delivery of antibiotics in the treatment of bone infections, see appendix), demonstrated that injection of BT2-minipeg-2 at 11 mg/kg, which is the molar equivalent of the amount of BT2-minipeg-2 contained in the BT2-minipeg-2 vancomycin conjugate, resulted in a dramatic accumulation of BT2-minipeg-2 in bone. Specifically, in samples taken 12 hrs after the last injection, the concentration of BT2-minipeg-2 was 235.0 ± 96.8 ng per gram of bone, while plasma levels were below the limit of detection. Most importantly, we did not observe gross anatomical or histopathological changes in the kidney, and biochemical assays confirmed normal kidney function in all BT2-minipeg-2 treated rats. Similarly, we did not observe any histological changes in the bone, and white blood cell (WBC) counts remained normal, the latter being indicative of normal bone marrow function. Thus, we conclude that BT2-minipeg-2 is not toxic and is likely to be a safe carrier for targeting at least some antibiotics, and potentially our small molecule inhibitors of *sarA* (see below) to bone. In fact, even absent such an inhibitor, this enhanced antibiotic targeting alone could have a tremendous impact on current clinical practice in that it could minimize the extent of surgical debridement required or perhaps in some cases even eliminate the need for surgical debridement entirely.

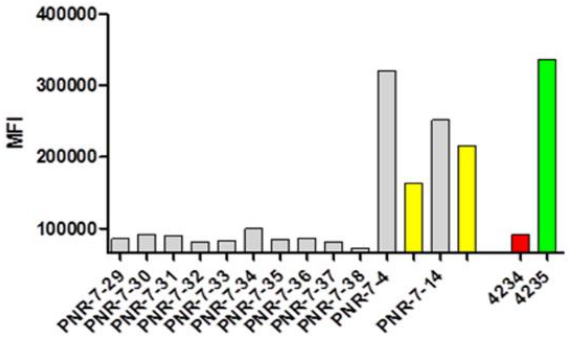
Finally, while our results with daptomycin suggest that BT2-minipeg-2 is the preferred carrier by comparison to BT2 itself, this will not necessarily prove to be the case with other antibiotics. Thus, we thought it was also important to test the toxicity of BT2 itself. These experiments were done as described above and in the submitted Albayati et al. manuscript (see appendix) except that rats were given a single oral dose of 500 mg per kg of BT2. Kidneys and the right and left tibia were harvested 7 days after this single oral dose was administered. The results of these studies also demonstrated that BT2 was not associated with significant toxicity in either the kidney or bone (data not shown). However, the more important point is that even 7 days after a single oral dose of BT2, the levels of BT2 in the bone were 326.3 ± 103.3 ng per gram of bone. We have not yet examined the accumulation of BT2-minipeg-2 in the bone after oral administration because we wanted to mimic the route of administration employed by Karau et al. (2013), but this raises the intriguing possibility that our bone targeting agent could be used in an oral antibiotic formulation, thus greatly enhancing ease of use and potentially patient compliance.

Task 4: Optimize previously identified compounds based on inhibition of *sarA*-mediated regulation. In addition to our studies focusing on conventional antibiotics and the ability to optimize their conjugation to our BT agents, we have continued to carry out our screen for inhibitors of *sarA*-mediated expression and/or function. This is based on our demonstration that mutation of *sarA* limits biofilm formation to a degree that can be correlated with increased antibiotic susceptibility (Weiss et al., 2009, Atwood et al., 2016), limits cytotoxicity for both osteoblasts and osteoclasts (Loughran et al., 2016), and limits overall virulence in animal models of both bacteremia (Rom et al., 2017) and osteomyelitis (Loughran et al., 2016). We have also demonstrated that this is due in large part to the increased production of extracellular proteases in *sarA* mutants and the resulting decrease in the accumulation of multiple surface-associated and extracellular virulence factors (Beenken et al., 2014, Loughran et al., 2014, Tsang et al., 2008, Zielinska et al., 2012). *S. aureus* encodes 10 known extracellular proteases encoded by individual genes (aureolysin; *aur*) or organized into each of 3 operons (*scpAB*, *splA-F*, and *sspABC*), We generated reporter constructs consisting of the promoters from these genes/operons fused to superfolder green fluorescent protein (sGFP). Subsequent studies confirmed that, by comparison to the isogenic parent strain, fluorescence was in fact increased with all four reporters when they were present in *sarA* mutants.



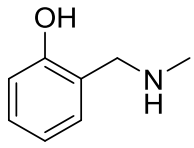
Fluorescence intensity is shown in each of two *S. aureus* strains (LAC and UAMS-1, designated here as U1) and their isogenic *sarA* mutants. The specific protease gene/operon promoter fused to the gfp reporter is shown below the graph. While expression from all 4 promoters was increased in the *sarA* mutant generated in both strains, we chose to use the *scpA::gfp* reporter in our primary screen based on the fact that fluorescence was highest in both strain with this reporter, thus providing us with an extended dynamic range.

Using the *scpA::gfp* reporter, we initiated a screen of a small molecule library in an effort to identify compounds that increase fluorescence in the wild-type parent strain (red or strain 4234 in the graph below) to a level comparable to that observed in the corresponding *sarA* mutant (green or strain 4235 in the graph below). As a control to account for the fact that some compounds have inherent fluorescent properties, we also assayed individual compounds in the absence of the reporter construct (yellow). The objective of this screen is to identify a compound in which fluorescence is increased in the parent strain in the presence of the compound to a level at least 25% of that observed in the *sarA* mutant in the absence of the same compound after accounting for any intrinsic fluorescence of the test compound itself. In the graph shown below, **PNR-7-4** is an example of such a compound, while **PNR-7-14** has been excluded from further study owing to its intrinsic fluorescence relative to the overall increase in fluorescence observed using our *scpA::gfp* reporter.

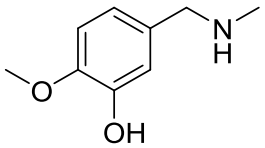


To date, we have screened 1,704 compounds, all in triplicate. Of these, we have identified 32 “hits” that meet the 25% benchmark described above. This is a “hit rate” of ~1.9%. Because these 32 compounds were identified in independent experiments done on different days over a long period of time, we are now re-screening all 32 in order to verify our results and make direct comparisons between each of these compounds. This will allow us to prioritize compounds for further analysis based on the degree to which they enhance fluorescence with the *scpA::gfp* reporter relative to each other. The highest priority compounds will validated using secondary assays including biofilm formation itself, the production of SarA in the parent *S. aureus* strain in the presence of the compound of interest, and relative antibiotic susceptibility in the context of an established biofilm (see previous progress report).

In addition to focusing more closely on the 32 compounds identified to date, we are continuing to screen additional compounds in our existing library as well as analogues of promising compounds generated based on examination of structure activity relationships (SAR). We are also examining the activity of anti-biofilm compounds reported in the literature. For example, Balamurugan et al. (2017) described the synthesis of a small molecule 2-[(methylamino)methyl]phenol (**PNR-10-117**) which was specifically targeted toward SarA based on *in silico* studies. These authors concluded that “the SarA targeted inhibitor showed negligible antimicrobial activity and markedly reduced the minimum inhibitory concentration of conventional antibiotics when used in combination”, and this is entirely consistent with the objectives of the work we are carrying out as part of this task. However, we synthesized this compound and were unable to verify this conclusion based on the results observed in our reporter assay or based on biofilm formation itself (data not shown). This could be a function of the manner in which our respective assays were done, but we would emphasize in this respect that the results observed in the assay we use to assess biofilm formation *in vitro* have been consistently verified with respect to also being relevant under *in vivo* conditions. Additionally, the degree of biofilm inhibition reported by these authors never exceeded 75-80%, and there was no difference in the levels of *sarA* mRNA in the presence or absence of this compound. This could be interpreted to mean that the compound functions at the post-transcriptional level, which would be an important and relevant observation, but even in this case we maintain the belief that the level of inhibition reported is likely to prove insufficient to have a significant therapeutic effect *in vivo*. In the context of our ongoing review of the literature as a means of identifying and optimizing promising inhibitors, it should also be noted that in at least some of these reports, putative inhibitors were also tested at very high concentrations up to 2 mg per ml (Arya et al., 2015), Finally, the enhanced antibiotic efficacy reported by these authors in the presence of their inhibitor was antibiotic specific and was not tested in the context of a biofilm. Based on all of these considerations, we are confident that the results we observed are indicative of the therapeutic potential of this compound. That said, we have synthesized an analogue of **PNR-10-117**, 2-methoxy-5-((methylamino)methyl)phenol (**PNR-10-118A**), and are in the process of testing the efficacy of this compound as both an inhibitor of biofilm formation and an inhibitor of SarA production and/or function.

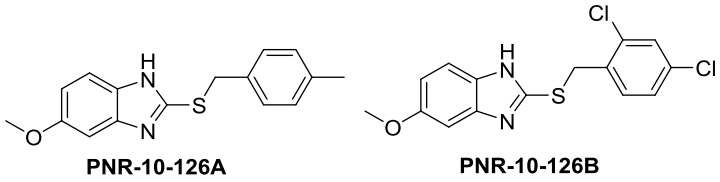


PNR-10-117

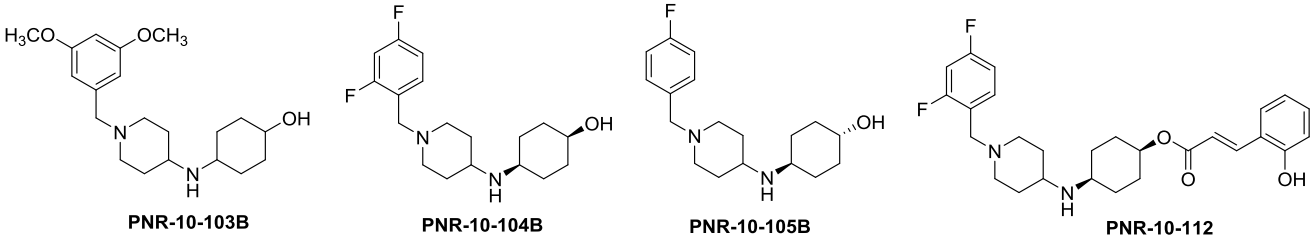


PNR-10-118A

We also synthesized benzimidazole derivatives based on the report of Sambanthamoorthy et al. (2011), who reported the synthesis of a novel benzimidazole molecule, named antibiofilm compound 1 (ABC-1; **PNR-10-126A**) and ABC-2 (**PNR-10-126B**), that were found to prevent biofilm formation in multiple Gram-negative and Gram-positive pathogens including *S. aureus*. However, neither of these compounds exhibited inhibitory effects as assessed using our *in vitro* assay procedures. We are very cognizant of the fact that we do not want to overlook a promising *sarA* inhibitor based on assay conditions, but as noted above we are fully confident in our assay procedures based on many years of experience, which clearly demonstrate that the results of our *in vitro* assays translate to *in vivo* efficacy.

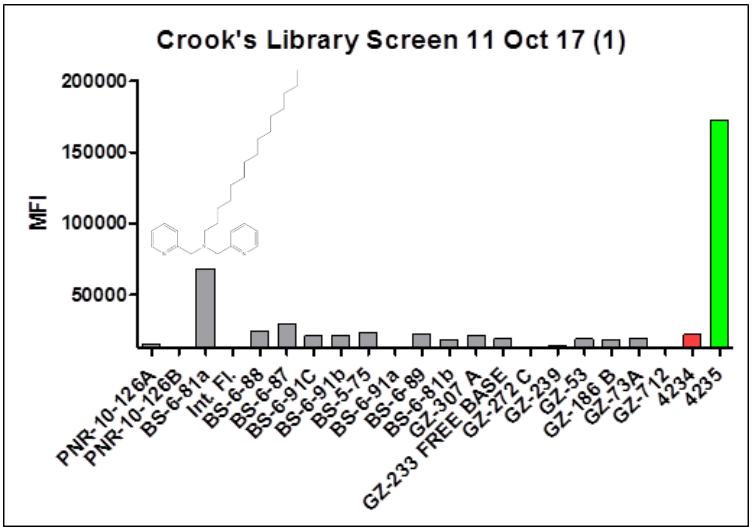


Finally, on the basis of our ongoing literature review, specifically a report describing a biofilm inhibitor (SarABI^{M}) that was reported to be effective against multidrug resistant *S. aureus* associated with gestational urinary tract infections (Balamurugan et al., 2015), we synthesized additional compounds (**PNR-10-103B**, **PNR-10-104B**, **PNR-10-105B**, and **PNR-10-112**) to confirm and perhaps even enhance their inhibitory activity against SarA and biofilm formation; further study of these compounds is pending.



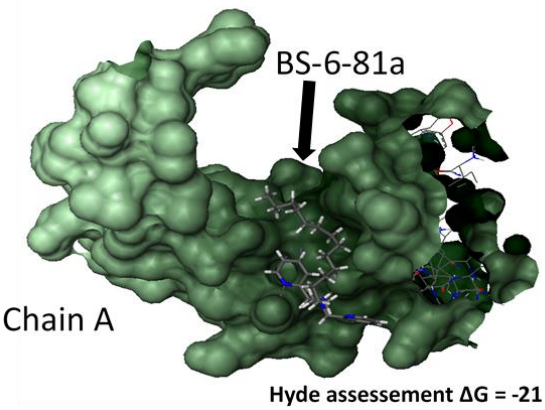
Task 5: Screen additional compounds to identify small molecule inhibitors of *sarA*-mediated regulation. Our small molecule library contains ~3,000 compounds, and as noted many of these remain to be screened in our primary reporter assay. This work is ongoing, but in addition to this we have begun to generate analogues of both previous "hits" from our library screen and compounds identified from our ongoing literature survey. In addition, we have begun molecular modeling studies as further confirmation of the results of our screen of these compounds and, importantly, to potentially provide an indication of whether promising compounds function at the level of inhibiting transcription of *sarA* and thereby limiting the production of SarA or, alternatively, act at the post-translational level by inhibiting the function of SarA.

As an example of our use of molecular modeling, we developed one analogue (**BS-6-81a**) based on the report of Goswami et al. (2014) and demonstrated that this compound resulted in a significant increase in fluorescence from our *scpA::gfp* reporter in the absence of detectable intrinsic fluorescence:

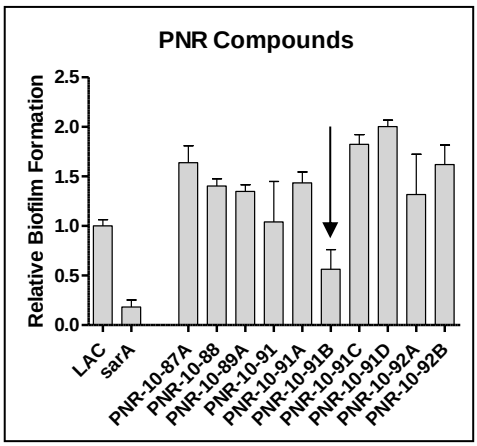


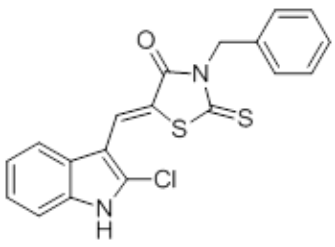
Results of *scpA::gfp* reporter assay with analogues of compounds contained in our primary library. BS-6-81A (chemical structure shown in inset) was the most promising compound identified in these assays and has now been synthesized in amounts sufficient to pursue our secondary screens.

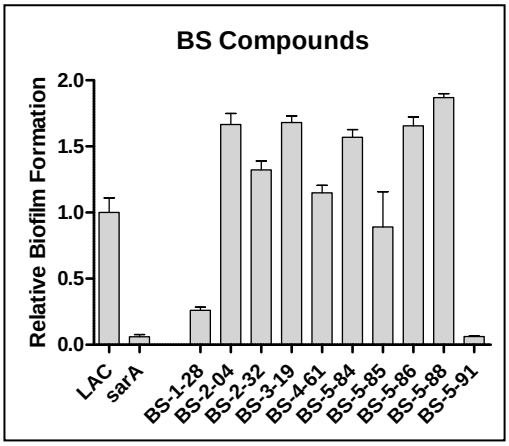
In addition to pursuing secondary assays with this compound, which are currently underway, we also carried out molecular modeling studies, the results of which indicate that **BS-6-81a** binds to the dimer interface of the SarA molecule as illustrated below. This is an important observation given that SarA is known to function in its capacity as a DNA-binding protein as a dimer (Rechtin et al., 1999). Thus, these results suggest that **BS-6-81a** may act by preventing the binding SarA to its DNA targets. If this is true, then it would be anticipated that **BS-6-81a** would inhibit biofilm formation but would not necessarily have an impact on the production of SarA itself.



Interestingly, we have also identified analogues that inhibit biofilm formation despite the fact that they were not associated with increased fluorescence in our primary screen. We chose to utilize this screen based on our results confirming that mutation of *sarA* results in a greater decrease in biofilm formation, and a greater increase in protease production, than mutation of any other *S. aureus* regulatory gene we have examined (Atwood et al., 2015, Rom et al., 2017), but it also impacts other *S. aureus* phenotypes that have been implicated in biofilm formation (Beenken et al., 2004, Beenken et al., 2012). Thus, while it makes experimental sense to use a reporter assay in the context of the kind of high-throughput required to screen a library, it is not necessary to do so when examining a more limited set of compounds that were either derived from the literature or are analogues of previously identified compounds. Specific examples of compounds which we found limited biofilm formation but were not identified using our reporter assay are shown below. We would note, however, that **BS-1-28** and **BS-5-91** (right panel) were found to have antibacterial activity. While potentially worth investigating, determining whether these compounds have potential as antibiotics is outside the scope of our grant. In contrast, one compound **PNR-10-91B** synthesized based on an existing patent aimed at identifying inhibitors of bacterial biofilm formation (WO 2009142720 A1) had no antibacterial activity but did significantly limit biofilm formation (left panel, arrow). Based on this, we are currently pursuing studies to investigate the mechanistic basis by which **PNR-10-91B** inhibits biofilm formation and whether it does so in diverse clinical isolates of *S. aureus* (Task 6).







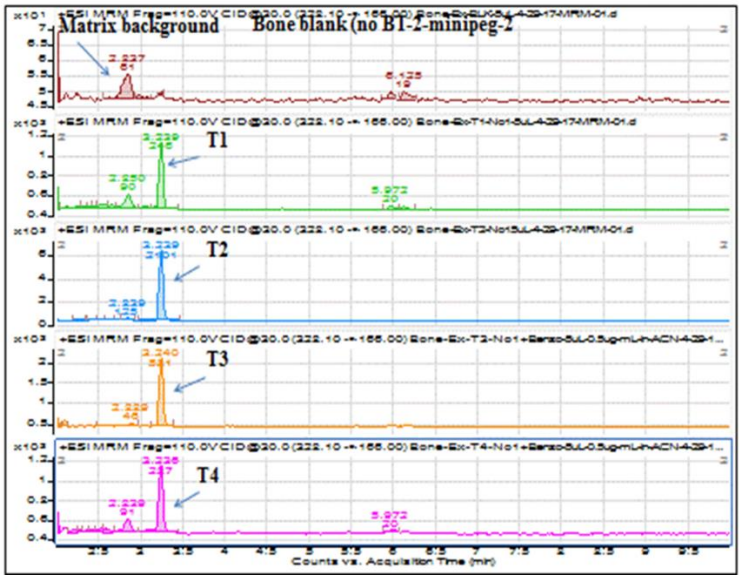
Chemical structure of **PNR-10-91B**

Task 6: Expand the screen of small molecule inhibitors to include additional staphylococcal strains and species. We have included the MRSA strain LAC and the MSSA strain UAMS-1 in our experiments because they are genetically and phenotypically distinct by comparison to each other in a fashion that extends far beyond the methicillin-resistance status. Based on this, we believe that identifying *sarA* inhibitors that are effective against both of these strains will greatly increase the likelihood that the results we observe will have broad utility in the context of diverse strains of *S. aureus*. As these inhibitors are identified and then verified in our secondary assays, we will then extend our studies to include additional strains of *S. aureus* as well as representative coagulase-negative species beginning with *S. epidermidis*.

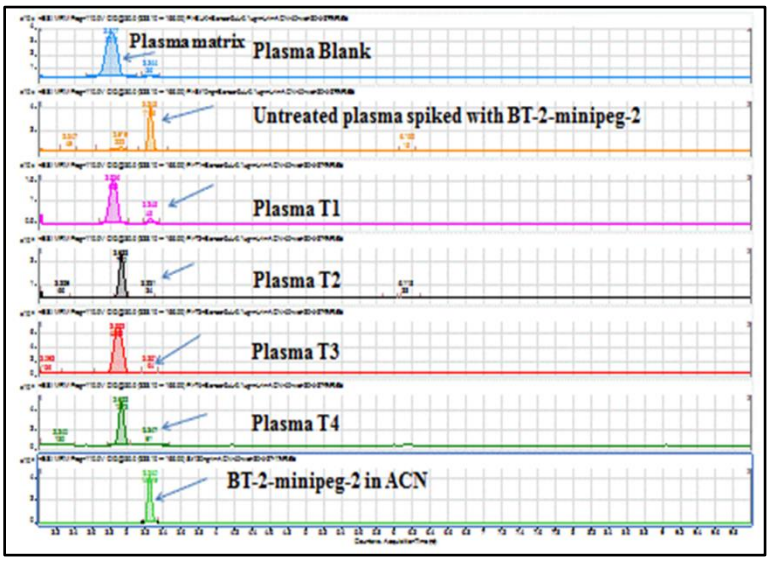
Task 7: Evaluate conjugation of the most promising *sarA* inhibitor to BT-2-minipeg-2. To date, we have not yet identified a *sarA* inhibitor that meets all of our selection criteria, thus precluding us from pursuing these studies at this time. We recognize that identifying an effective *sarA* inhibitor is, by definition, not guaranteed, but we believe our comprehensive approach as detailed above puts us in a unique position to accomplish this task and to do so in the relatively near future. Additionally, given the severity of the clinical problem of bone infections following traumatic injury, we believe the effort is not only well justified but in fact necessary.

Task 8: Evaluate *in vivo* pharmacological properties of the most promising *sarA* inhibitor and its BT-2-minipeg-2 conjugate. These studies are also pending for the same reason cited above in the context of Task 7.

Task 9: Evaluate the efficacy of the most promising small molecule inhibitor and the most promising antibiotic *in vivo* with and without conjugation to BT-2-minipeg-2. As detailed above we are now in a position to move forward with *in vivo* studies employing our BT2-minipeg-2 ciprofloxacin and daptomycin conjugates, and in fact these studies are currently underway. This required us to develop and optimize an analytical method to detect daptomycin in the bone and in plasma. This was accomplished using an Agilent triple quadrupole mass spectrometer. The mass spectrometer was operated in the positive electrospray ionization multiple reaction monitoring (MRM) mode. BT2-minipeg was analyzed using an Alltech Altima C18 column (150 mm×3.2 mm, 5.0 µm) (Grace Discovery Sciences, IL, USA) fitted with an Alltech Altima C18 guard column (7.5 X 3.0 mm, 5 µm) (Grace Discovery Sciences, IL, USA). The mobile phase consisted of water with 0.1% formic acid as solvent A and acetonitrile (neat) as solvent B. Analysis was carried out using a gradient method. MRM transitions monitored were as follows: m/z 328.1/166.0 for BT2-minipeg and m/z 183.1/105.1, m/z 183.1/77.1 for benzophenone, which was spiked into all samples as an internral control. Using this method, we confirmed that significant amounts of BT-2-minipeg were detected in the left tibia (235.0 ± 96.8 ng/g) compared to no significant concentrations in the plasma:



Chromatograms of BT2-minipeg-2 showing results observed in the tibia in an untreated control (top row) vs. rats treated with BT2-minipeg-2 (T1-T4).



Chromatograms of BT2-minipeg-2 results observed in plasma in an untreated animal (top row), plasma spiked with BT2-minipeg-2 (plasma standard, 2nd row), and chromatograms from four rats treated with BT2-minipeg-2 (T1-T4). Bottom row shows a chromatogram of a BT2-minipeg-2 standard prepared in acetonitrile.

OPPORTUNITIES FOR PROFESSIONAL DEVELOPMENT

This is an interdisciplinary collaborative project between the microbiologists in the Smeltzer research group and the medicinal chemists/pharmaceutical scientists in the Crooks research group. Both have a great deal to learn from the other, and thus are benefitting greatly from their collaborative effort. This is significantly enhanced by combined lab meetings that include members of both groups. The Smeltzer research group also includes technicians and graduate students who are participating directly in various aspects of the project and thereby learning directly from members of both research groups. Every effort is also being made to publish the results of our studies in respected, peer-reviewed, open-access journals and to present these results at relevant scientific meetings. We believe this will increase our exposure on a national and international level and has the potential to lead to additional collaborative efforts that may ultimately further expand the clinical relevance and impact of our work.

DISSEMINATION OF RESULTS TO COMMUNITIES OF INTEREST

Results are being disseminated through publication respected, peer-reviewed, open-access journals and participation at relevant scientific meetings.

PLANS TO ACCOMPLISH OUR GOALS

We will continue to screen for inhibitors of *sarA*, optimize the chemistry and function of such inhibitors, optimize conjugation of these inhibitors and previously prioritized antibiotics to BT2-minipeg-2, and ultimately undertake *in vivo* studies examining pharmacological properties and therapeutic efficacy.

IMPACT ON PRINCIPAL DISCIPLINE(S) OF THE PROJECT

The experiments we describe have not yet progressed to the point where we can carry out the studies necessary to 1) confirm the pharmacokinetic and bone targeting properties of alternative BT2-minipeg-2 antibiotics and/or *sarA* inhibitor conjugates; 2) demonstrate a lack of mammalian cell and host systemic toxicity and 3) evaluate the prophylactic and therapeutic efficacy of our BT2-minipeg-2 conjugates. However, we have made tremendous strides toward these goals, and we are confident that we will ultimately be successful in accomplishing all of these tasks. Moreover, we are equally confident that this will have a tremendous impact on the standard of care in the specific context of enhancing the prevention and treatment of orthopaedic infections associated with traumatic injury.

IMPACT ON OTHER DISCIPLINES

Although we have nothing specific to report in this regard, our studies aimed at optimizing the use of BT2-minipeg-2 as a bone targeting agent in the context of antibiotics could have broad applicability to the delivery of other types of bioactive agents to the bone, thereby opening up the possibility of developing combination therapies that exploit our bone targeting agent not only to prevent and/or treat infection but also potentially to facilitate bone regeneration. Indeed, while outside the scope of this grant, we have already began to investigate this possibility using funds from other sources. We would emphasize that no funds from the currently funded project are being used for these studies as this would represent a change in scope, but we are nevertheless confident that optimization of BT2-minipeg-2, or BT2 itself as dictated by the results of our ongoing experiments (see above), offer tremendous promise in this regard.

IMPACT ON TECHNOLOGY TRANSFER

Nothing to report.

IMPACT ON SOCIETY BEYOND SCIENCE AND TECHNOLOGY

Nothing to report.

CHANGES IN APPROACH AND REASONS FOR CHANGE

Nothing to report.

ACTUAL OR ANTICIPATED PROBLEMS

Nothing to report.

CHANGES THAT IMPACTED EXPENDITURES

Nothing to report.

SIGNIFICANT CHANGES IN HUMAN SUBJECTS, VERTEBRATE ANIMALS, BIOHAZARDS, AND/OR SELECT AGENTS

Human subjects are not applicable. There have been no changes with respect to biohazards, select agents, or vertebrate animals since the previous progress report. As detailed in the previous report, all changes involving vertebrate animals received prior approval from both the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences (UAMS) and the Animal Care and Use Review Office (ACURO) of the U.S. Army Medical Research and Materiel Command (USAMRMC).

PUBLICATIONS, CONFERENCE PAPERS, PRESENTATIONS

1. **Meeker, D.G., Loughran, A.J., Beenken, K.E., Spencer, H.J., Lynn, W.B., Mills, W.B., and Smeltzer, M.S.** 2016. Evaluation of antibiotics effective against methicillin-resistant *Staphylococcus aureus* based on efficacy in the context of an established biofilm. Antimicrobial Agents and Chemotherapy, 60:5688-5694. PMCID: PMC5038242
2. **Albayati, Z.A.F., Sunkara, M., Schimdt-Malan, S.M., Karau, M.J., Morris, A.J., Steckelberg, J.M, Patel, R., Breen, P., Smeltzer, M.S., Taylor, K.G., Merten, K.E., Pierce, W.M., and Crooks, P.A.** 2015. Use of a novel bone-targeting agent for the enhanced delivery of vancomycin to bone. Antimicrobial Agents and Chemotherapy. 60:1865-1868. PMCID: PMC4776008.
3. **Moon, S.H., Zhang, X., Zheng, G., Meeker, D.G., Smeltzer, M.S., and Huang, E.** Structure-activity relationship studies of linear lipopeptide paenipeptins with potential for eradicating biofilms and sensitizing Gram-negative bacteria to rifampicin, submitted to Journal of Medicinal Chemistry (see appendix).
4. **Albayati, Z.A.F., Bommaganni, S., Penthala, N., Post, G.R., Smeltzer, M.S., Crooks, P.A.** BT2-minipeg-2 is a safe carrier for the targeted systemic delivery of antibiotics in the treatment of bone infections, submitted to American Association of Pharmaceutical Sciences Journal (see appendix).

5. **American Association of Pharmaceutical Sciences (AAPS), 2017, San Diego, CA.** BT2-minipeg-2 is a safe carrier for the targeted systemic delivery of antibiotics in the treatment of bone infections.
6. **Military Health Science Research Symposium (MHSRS) 2016, Orlando, FL.** Enhancing the prevention and treatment of orthopaedic infections associated with traumatic injury.

WEBSITE/OTHER INTERNET SITES

Nothing to report.

TECHOLOGIES OR TECHNIQUES

Nothing to report.

INVENTIONS, PATENT APPLICATIONS, AND/OR LICENSE

Huang E., Moon S.H., Zheng G., Zhang X., Smeltzer M.S., Meeker D.G. 2017. Linear Polypeptide Paenipeptins and Methods of Using the Same. U.S. Provisional Application No. 62/541,200

OTHER PRODUCTS

Nothing to report.

INDIVIDUALS WHO HAVE WORKED ON THE PROJECT

Personnel	Role	Percent Effort
Mark Smeltzer	PI	20%
Peter Crooks	Co-Investigator	10%
Albayati Zaineb	Post-doctoral Research Associate	50%
Narsimha R. Penthala	Research Instructor	50%
Karen Beenken	Research Associate Professor	50%
Daniel Meeker	Graduate Student	50%
Weston Blake Mills[1]	Laboratory Technician	50%
Shobanbabu Bommagani	Post-doctoral Research Associate	100%

CHANGES IN ACTIVE SUPPORT OF PD/PI

Nothing to report since the previous annual progress report.

OTHER ORGANIZATIONS INVOLVED

Not applicable.

LITERATURE CITED

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APPENDIX

Albayati et al., manuscript submitted.

Moon et al., manuscript submitted.

BT2-MINIPEG-2 IS A SAFE CARRIER FOR THE TARGETED SYSTEMIC DELIVERY OF ANTIBIOTICS IN THE TREATMENT OF BONE INFECTIONS

Zaineb AF Albayati[1], Shobanbabu Bommagani[1], Narsimha Penthala[1], Ginell R Post[2], Mark S Smeltzer[3] and Peter A Crooks[*1]

[1]Department of Pharmaceutical Sciences, College of Pharmacy, [2]Department of Clinical Pathology, College of Medicine, and [3]Department of Microbiology & Immunology, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, AR 72205

*Corresponding author at: College of Pharmacy, University of Arkansas for Medical Sciences, Little Rock, AR, USA (PAC), pacrooks@uams.edu

Key words: BT2-minipeg-2, BT2peg, vancomycin, rat, plasma, bone, kidney, nephrotoxicity, pharmacokinetics

ABSTRACT

Purpose. BT2-minipeg-2 (BT2peg) is a novel bone targeting agent. In a previous study, BT2peg was conjugated to vancomycin (BT2peg-vancomycin) and administered intravenously (IV) or by intraperitoneal (IP) injection to Albino Wistar male rats. By comparison to the molar equivalent of unconjugated vancomycin administered in the same fashion, elevated levels of BT2peg-vancomycin were observed in plasma and in the bone. However, analysis of plasma revealed elevated levels of serum creatinine and blood urea nitrogen (BUN), and decreased levels of serum albumin. Marked nephrotoxicity was also evident both by gross examination and histological analysis. The objective of this study was to determine whether BT2peg contributed to the toxicity observed with the BT2peg-vancomycin conjugate.

Methods. 11 mg/kg of BT2peg, which is the molar equivalent of the amount of the BT2peg component previously examined in the form of the BT2peg-vancomycin conjugate, was formulated in phosphate buffered saline and injected IP into four Albino Wistar male rats weighing 200-250 gm. Injections were done twice daily for 21 days (42 doses). Rats were humanely euthanized 12 hrs after the last dose and the kidneys, plasma and right and left tibia collected. BT2peg concentrations were determined using a novel LC/MS/MS analytical method. Kidneys were examined histologically, while kidney function was evaluated based on BUN, plasma levels of creatinine and albumin. Total white blood cell count (WBC) was also determined to assess leukocytosis.

Results. The average percent change in body weight over the course of the 21 day treatment period did not differ significantly between untreated rats and rats treated with BT2peg. There was also no significant difference in average kidney weight between treated and untreated rats (2.50 ± 0.22 g and 2.37 ± 0.35 gm, respectively). Kidneys from both groups also exhibited no gross or histopathologic abnormalities. Chemical studies showed plasma levels of 22.1 ± 2.5 and 23.0 ± 0.9 mg/dl for BUN, 3.4 ± 0.02 and 3.5 ± 0.01 g/dl for albumin, and 0.5 ± 0.01 and 0.6 ± 0.01 mg/dl for creatinine in untreated and BT2peg-treated rats, respectively.

None of these differences were statistically significant. Total white blood cell counts were 8.9 ± 0.9 x 10^3 and 9.3 ± 0.8 x 10^3/μL for untreated and BT2peg-treated rats, respectively. This difference was also not statistically significant, thus confirming the absence of leukocytosis. Significant amounts of BT2peg were detected in the left tibia (235.0 ± 96.8 ng/g) while BT2peg was undetectable in plasma. Examination of the right tibia from BT2peg treated animals indicated no significant histopathologic abnormalities.

Conclusion. These results demonstrate that BT2peg has no nephrotoxicity and does not cause histopathological changes in the bone despite localization to bone in high concentrations. Additionally, BT2peg was rapidly cleared from the plasma. This suggests that the nephrotoxicity observed with BT2peg-vancomcin was due to elevated concentrations of vancomycin in the plasma and not to BT2peg itself. Although the nephrotoxicity associated with BT2peg-vancomycin emphasizes the need to carefully evaluate PK properties of BT2peg conjugates, we conclude that BT2-minipeg-2 is a safe compound that can be used for the targeted delivery of at least some antibiotics and perhaps other bioactive agents for the treatment of bone infections.

INTRODUCTION

Osteomyelitis is a serious inflammatory condition of bone that is most often associated with bacterial infection. The primary bacterial pathogen both in terms of its predominance as a causal agent and the severity of the resulting infection is *Staphylococcus aureus* (Cierny, 2011). Treatment of these infections is extremely challenging owing in part to the increasing prevalence of *S. aureus* strains resistant to the methicillin class of antibiotics (Chambers and DeLeo, 2009). However, even absent such resistance issues, conventional antibiotic therapy has limited effectiveness owing to multiple factors including a compromised local vasculature, the ability of the offending bacterial cells to invade and survive in host cells including osteoblasts, and formation of a biofilm, the presence of which confers a therapeutically-relevant degree of intrinsic resistance to both conventional antibiotics and host defense systems (Brady et al. 2008, Cierny, 2011, Josse et al. 2015).

Despite the development of a number of newer antibiotics with efficacy against methicillin-resistant *S. aureus* (MRSA), vancomycin remains the antibiotic of choice in the clinical treatment of bone and joint infections (Darley and MacGowan et al, 2004; Morata et al., 2015, Rubinstein and Keynan, 2014; Lew and Waldvogel, 2004). However, vancomycin is a large, hydrophilic molecule that exhibits poor penetration into bone resulting in low bone bioavailability when administered systemically (Massias, 1992; Martin, 1994). Thus, it is most often necessary to administer high doses of vancomycin for long periods of time, and even then eradication of the infection most often requires surgical debridement to remove all infected bone and associated tissues (Cierny, 2011). Such prolonged vancomycin treatment is also associated with a number of adverse consequences. These include the emergence of *S. aureus* strains that exhibit intermediate but therapeutically relevant levels of resistance to vancomycin (vancomycin intermediate *S. aureus* or VISA) (Hiramatsu et al., 2015) and nephrotoxicity, which can preclude its use in patients with poor kidney function and those requiring life-long

suppressive antibiotic therapy (Rodvold and McConeghy, 2014). At present, the primary means of overcoming these limitations is the use of localized, matrix-based antibiotic delivery, most often following surgical debridement (Lew and Waldvogel, 2004). Thus, there is an urgent need for improved methods for the more effective systemic delivery of antimicrobial agents to bone.

~~developed~~ BT2-minipeg-2 (BT2peg) was developed as a novel bone targeting agent based on its high affinity for bone calcium and hydroxyapatite and a specific capacity to accumulate in bone when administered systemically (Nasim et al 2010; Pierce et al, 2008, Pierce et al, 2011). Karau et al (2013) demonstrated that, under standard *in vitro* test conditions, BT2peg conjugated to vancomycin has similar activity to vancomycin itself against MRSA, indicating that conjugation of vancomycin to BT2peg does not affect its antimicrobial activity. Moreover, treatment of experimental osteomyelitis with vancomycin and the molar equivalent of BT2peg-vancomycin using the same dosing regimen confirmed that BT2peg-vancomycin exhibits enhanced therapeutic efficacy by comparison to vancomycin alone (Karau et al., 2013). We also confirmed by pharmacokinetic (PK) analysis that BT2peg-vancomycin significantly facilitates the accumulation of vancomycin in bone (Albayati et al, 2015).

While promising, these earlier studies also demonstrated that systemic administration of BT2peg-vancomycin in rats was also associated with high plasma concentrations, elevated levels of serum creatinine and blood urea nitrogen (BUN), decreased serum albumin, leukocytosis, and severe nephrotoxicity (Karau et al., 2013). Because vancomycin is known to exhibit kidney toxicity **(**Filippone et al, 2017), we hypothesized that these adverse consequences were due to the altered PK of BT2peg-vancomycin resulting in its prolonged accumulation in plasma and consequently exacerbation of the nephrotoxicity of vancomycin. To test this hypothesis, the present study was aimed at determining whether BT2peg itself, when administered systemically, contributes to the previously observed nephrotoxicity of BT2peg-vancomycin (Karau et al, 2013).

MATERIALS AND METHODS

Chemicals. All chemicals used in this study were of LC/MS grade or equivalent quality. Acetonitrile, methanol, formic acid, and normal saline were obtained from Fisher Scientific (Pittsburgh, PA, USA). Benzophenone was obtained from Sigma-Aldrich (St. Louis, MO, USA). Heparin sodium injection (10,000 USP units/mL) was purchased from Baxter Healthcare Corporation (Deerfield, IL, USA).

Animal experimental protocol. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences (UAMS) and the Animal Care and Use Review Office (ACURO) of the U.S. Army Medical Research and Materiel Command (USAMRMC). BT2peg was administered at a concentration of 11 mg/kg, which is the molar equivalent of the amount of BT2peg present in the BT2peg-vancomycin conjugate. BT2peg was formulated in phosphate buffered saline (PBS) and delivered by intraperitoneal (IP) injection into each of four Albino Wistar male rats (200-250 gm, Charles River, Wilmington, MA). Administration was done twice daily for 21 days for a total of 42 doses, which is the most intensive administration protocol employed by Karau et al (2013). Twelve hrs after the last dose, rats were humanely euthanized using CO_2 and blood, kidneys and the right and left tibia harvested from each rat. Blood was collected via cardiac puncture and placed into sodium heparin blood collection tubes for hematology. Plasma was obtained by centrifugation at 10,000 rpm at room temperature (RT) for 5 min. Samples were stored at -80^{o}C prior to analysis.

Histological and hematological analysis. Kidneys from untreated and BT2peg-treated rats were collected, weighed, observed for abnormalities in size and color, and fixed in neutralized buffered formalin (NBF) for 24 h prior to histological analysis. Histological analysis was performed using Hematoxylin and Eosin (H&E) and Periodic acid–Schiff (PAS) stained sections. The left and right tibia were cleaned of soft tissues, washed with PBS and weighed. Right tibia samples were fixed in NBF for 24 h and decalcified using 10% EDTA (pH 7.0) prior to

histological analysis. Plasma and the left tibia were used for quantitative determination of BT2peg concentration by LC/MS as detailed below. Hematological analysis of blood was performed to determine white blood cell (WBC) count and blood urea nitrogen (BUN) levels. UV/visible colorimetric analyses was used to determine plasma creatinine and albumin levels as previously described (Wybenga et al., 1971; Krishnegowda et al., 2017, Pinnell, 1978).

Preparation of plasma samples for LC/MS analysis of BT2peg levels. A hundred µL of plasma from untreated and BT2peg-treated rats was spiked with benzophenone (10 µL of a 0.1 µg/mL solution in acetonitrile) as an internal standard. Protein precipitation was performed by adding 400 µL of acetonitrile. After 30 s of vortexing, the solution was centrifuged at 10,000 X g for 10 min at RT. The supernatant was evaporated to dryness under nitrogen and each pellet reconstituted with 60 µL of (acetonitrile:water, 40:20 µL) . Re-suspended pellets were vortexed, sonicated for 1 min, and centrifuged at 10,000 rpm for 10 min at RT prior to analysis by LC/MS as detailed below.

Preparation of bone samples and extraction procedure for analysis of BT2peg analysis. Stainless steel balls (3.5 mm, Next Advance Inc, Troy, NY, USA) were placed in a 5 ml tube along with the tibia, 0.5 mL hexane, and 1 mL of water. Bone samples were homogenized for 3 min using a Bullet Blender Storm 5 homogenizer (Next Advance, Inc. Troy, NY, USA). Ten µL (0.1 µg/mL) of benzophenone was added to 0.2 mL of each bone homogenate as an internal standard. Samples were vortexed and extracted with 600 µL ACN followed by the addition of 400 µL water. Samples were then vortexed for 30 s and centrifuged at RT for 10 min at 10,000 rpm. Supernatants were then processed as described above for analysis by LC/MS.

LC/MS analysis of BT2peg in plasma and bone samples. Five µL of samples extracted from plasma and bone were used to assess BT2peg levels using an Agilent LC/MS Triple Quad 6410 instrument (Santa Clara, CA, USA) operated in the positive electrospray ionization (ESI) in the multiple reaction monitoring (MRM) mode with optimal ion source settings

determined by standards of BT2peg and benzophenone as internal standard. A curtain gas of 20 psi, an ion spray voltage of 4000 V, an ion source gas1/gas2 of 35 psi and temperature of 300°C were employed in the collection of chromatographic data. Chromatographic separation was carried out with an Alltech Altima C-18 column (150 mm×3.2 mm, 5.0 µ) fitted with an Alltech Altima C-18 guard column (7.5 X 3.0 mm, 5µ, Grace Discovery Sciences, IL, USA). The mobile phase consisted of water added to 0.1% formic acid as solvent A and acetonitrile as solvent B. The separation was achieved using a flow rate of 0.8 mL/min and a gradient of 10 to 90% solvent B in solvent A for 3.50 min. Flow was maintained at 90% solvent B for the next 3.50 min and then equilibrated back to the initial 10% solvent B conditions over 3.20 min. BT2peg and the benzophenone internal standard (IS) exhibited retention times of 3.237 and 5.603 min, respectively (data not shown). MRM transitions monitored were m/z 328.1/166.0 for BT2peg, and m/z 183.1/105.1 and m/z 183.1/77.1 for benzophenone.

Histopathology. Kidney and bone samples were fixed in 10% NBF. Bone samples were decalcified in 10% EDTA buffer, pH 7.0. Tissues were then placed in cassettes and embedded in paraffin. Samples were then sectioned at 5 µM and stained with Haematoxylin and Eosin (H&E). For kidney sections, the periodic acid-Schiff (PAS) stain was also used to highlight the tubular brush border cells. Histologic sections prepared from the kidneys and right tibia were evaluated microscopically by a pathologist in a blinded fashion.

Statistical analysis. Data are presented as mean ± standard error of the mean (mean ± SEM). The data were analyzed for statistical significance using the unpaired Student's t-test with Welch's correction factor for unequal variances and $p \leq 0.05$ as the criterion of significance.

RESULTS

The percent change in body weight over the course of 21 days treatment for the untreated and BT2peg-treated groups were not statistically different ($p \geq 0.05$, Fig. 1). The kidneys from the untreated and the BT2peg-treated rats exhibited a deep maroon color and

were indistinguishable visually (Fig. 2), and their size and weight indicated no significant difference between the two groups (Table 1). Most importantly, histologic evaluation of the renal sections from BT2peg-treated and untreated rats showed unremarkable glomeruli, tubules, vessels and interstitium. There was also no histopathologic features of renal injury, including tubular dilatation, apical budding, brush border or tubular loss (Fig. 3). In addition, biochemical results indicated normal values for blood urea nitrogen (BUN) or plasma albumin and creatinine in both the untreated and BT2peg-treated animals (Table 1).

There was also no statistical difference in total white blood cell counts for the untreated and BT2peg-treated groups, which exhibited values of $8.9 \pm 0.9 \times 10^3$ and $9.3 \pm 0.8 \times 10^3/\mu L$, respectively. Significant amounts of BT2peg were detected in the left tibia (235.0 ± 96.8 ng/g), while BT2peg was undetectable in plasma. Microscopic examination of the right tibia from BT2peg-treated and untreated rats showed similar features, including intact cortical and paratrabecular bone with morphologically unremarkable trilineage hematopoiesis. There was no evidence of cellular or stromal injury in either experimental group (Fig. 4).

DISCUSSION

Previous studies employing a rat model of experimental osteomyelitis provided evidence that BT2peg-vancomycin delivered systemically by intravenous (IV) or IP injection exhibits greater therapeutic efficacy in the context of bone than an equivalent dose of vancomycin (Karau et al. 2013). However, the use of BT2peg-vancomycin was also associated with a profound change in pharmacokinetic (PK) profile characterized by a high plasma levels of BT2peg-vancomycin, decreased animal weight, increased kidney size, and severe tubulointerstitial nephritis. Subsequent chemical analysis also confirmed that administration of BT2peg-vancomycin resulted in elevated levels of serum creatinine and blood urea nitrogen (BUN) and decreased serum albumin. The purpose of this study was to determine whether BT2peg moiety itself contributed to these adverse effects. To this end, we administered 11

mg/kg of the BT2peg moiety, which is the molar equivalent of the BT2peg component of BT2peg-vancomycin, using the same dosing regimen (IP injection twice daily for 21 days) shown to enhance therapeutic efficacy in these earlier experiments (Karau et al., 2013).

The results demonstrate that administration of BT2peg itself is not associated with any of the adverse side effects observed in these previous studies. Specifically, there was no statistically significant difference between the untreated and BT2peg-treated experimental groups as assessed by weight loss, kidney size and overall morphology, histopathological changes in the kidney, or changes in blood urea nitrogen (BUN), albumin or creatinine levels. All of these results are consistent with the hypothesis that BT2peg itself was not responsible for the nephrotoxicity observed in the earlier studies (Karau et al., 2013).

In order to test this hypothesis as stringently as possible, we employed the maximum dosing regimen employed by Karau et al. (2013), which was twice daily for 21 days. In this respect it should be noted that nephrotoxicity was minimized if not eliminated using more conservative dosing regimens (every 12 hrs for 3.5 days followed by once daily every fourth day or once per week), but these more conservative regimens were not demonstrably associated with an enhanced therapeutic effect. It should also be noted that plasma levels of BT2peg-vancomycin were dramatically elevated using the more intensive dosing regimen and this was not the case with either of the more conservative dosing regimens (Karau et al., 2013). This suggests a direct correlation between plasma levels of BT2peg-vancomycin and nephrotoxicity.

It was suggested that the high plasma levels observed with the high dosing regimen were likely due to the pegylation component of the BT2peg-vancomycin formulation in that pegylation is known to increase drug half-life, although the possibility that release of BT2peg-vancomycin from the bone into the systemic circulation also contributed to the high levels observed in plasma could not be ruled out (Karau et al., 2013). We found that plasma levels of BT2peg were below the limit of detection despite the clear accumulation of BT2peg in the bone. While certainly not definitive, this suggests that the elevated plasma levels observed by Karau

et al. (2013) were due to altered PK parameters and reduced clearance of BT2peg-vancomycin rather than sustained release from the bone.

This is consistent with our previous PK studies demonstrating that vancomycin was first detectable in the blood 1 hr after administration and was cleared within 12 hr (Albayati et al., 2015). In contrast, plasma BT2peg-vancomycin was also first detected at 1 hr, but remained detectable for at least 168 h, demonstrating decreased total clearance (Cl_{tot}) from the body. In fact, a decrease of 13.5-fold in Cl_{tot} was displayed by BT2peg-vancomycin compared to vancomycin with a 14.7-fold increase in $t_{1/2}$ for the former, allowing for a 10.8-fold enhancement in the area under the curve (AUC) for BT2peg-vancomycin (Albayati et al, 2015). The later PK findings agree well with the data reported by Karau et al (2013) data. Thus, to the extent that the dosing regimen we employed resulted in high levels of BT2peg in the bone but not in plasma, this suggests that the high plasma levels observed with BT2peg-vancomycin are due to reduced clearance of BT2peg-vancomycin conjugate and not the BT2peg component itself.

Importantly, we not only confirmed the targeting efficiency of BT2peg in the context of bone, but also that its accumulation in bone is not associated with adverse bone pathology or leukocytosis. Thus, both histological and hematology results indicate normal bone marrow function. Overall, we conclude that BT2peg has tremendous potential as a safe and effective bone targeting agent and that the nephrotoxicity observed in earlier experiments is in fact a function of its conjugation to vancomycin, likely owing to prolonged persistence in the blood. This suggests that BT2peg could be used to enhance the systemic delivery of antibiotics other than vancomycin to bone. However, the results we report also emphasize the need to carefully evaluate PK parameters and potential toxicity of alternative BT2peg conjugates in addition to their therapeutic efficacy in the context of bone infection. **ACKNOWLEDGEMENTS**

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. None of the authors have financial interests to declare.

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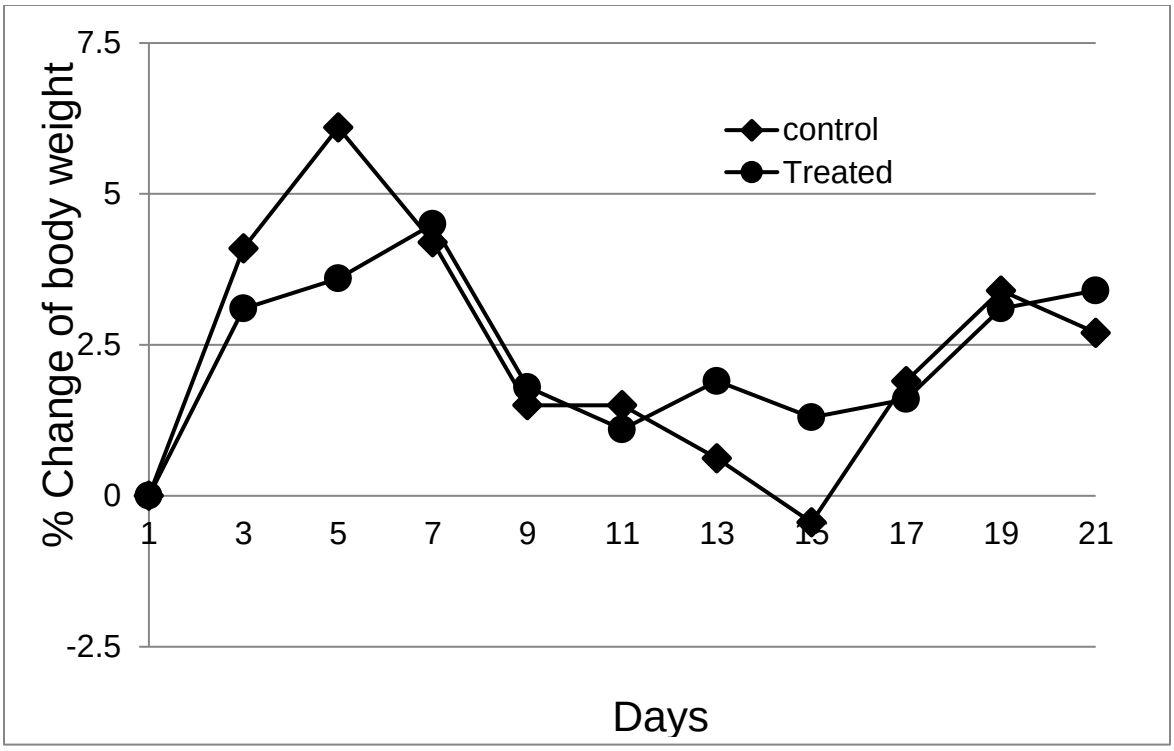


Fig. 1. Percent body weight change over the 21 course of treatment with BT2peg.

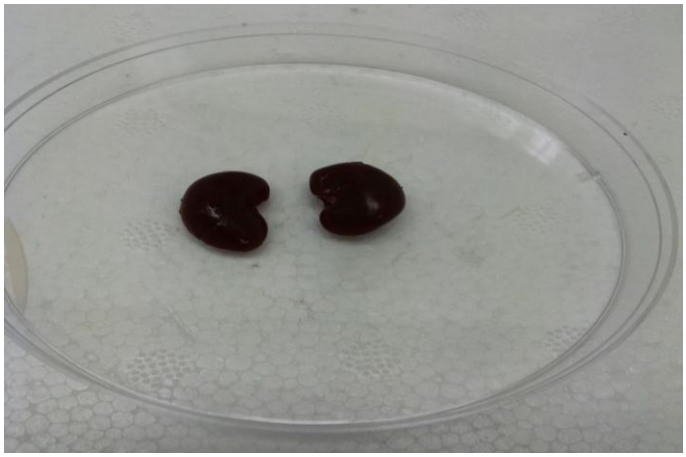
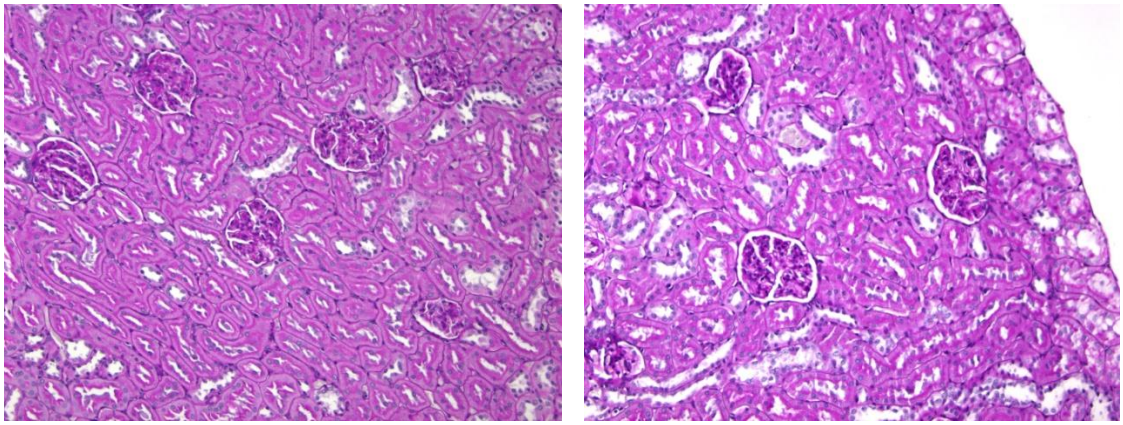


Fig. 2. Kidneys from untreated (left), and BT2peg-treated rats (right).

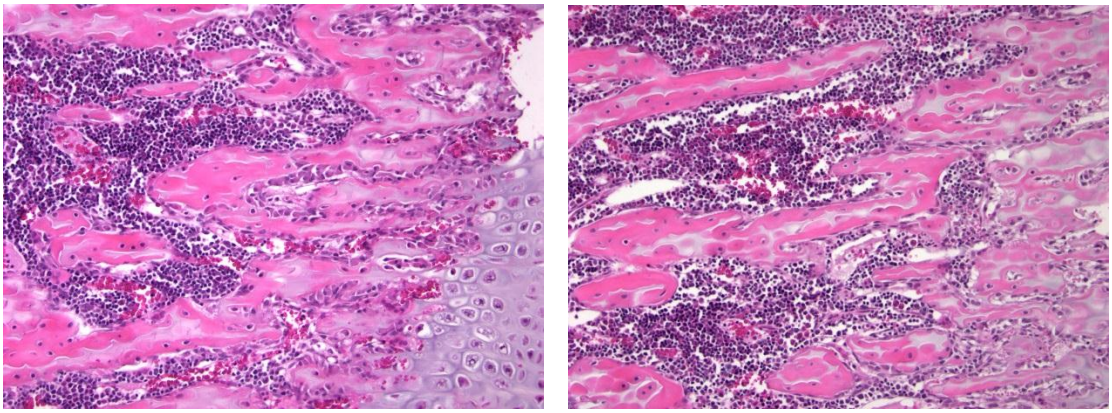
Table 1. Kidney weights, BUN, plasma albumin and creatinine values after treatment with BT2-peg2 at a dose of 11 mg/kg (the molar equivalent of BT2-peg2 used in BT2-peg2 vancomycin)

Group	Kidney weight gm	BUN mg/dl	Albumin gm/dl	Creatinine mg/dl
Untreated	2.5 ± 0.22	22.1 ± 2.5	3.4 ± 0.02	0.5 ± 0.01
BT2peg-treated	2.4 ± 0.35	23.0 ± 0.9	3.5 ± 0.01	0.6 ± 0.01



A B

Fig. 3. Periodic acid–Schiff (PAS) stained histopathologic sections from A) untreated and B) BT2peg-treated kidneys. There was no discernible evidence of microscopic glomerular or renal tubular damage as evidenced by tubular dilatation, apical budding, or brush border and tubular loss. The absence of demonstrable histopathology was also confirmed by H&E staining (data not shown).



A B

Fig. 4. Hematoxylin and Eosin stained sections of the right tibia from representative A) untreated and B) BT2peg-treated rats.

Novel linear lipopeptide paenipeptins with potential for eradicating biofilms and sensitizing Gram-negative bacteria to rifampicin and clarithromycin

Sun Hee Moon[1], Xuan Zhang[2], Guangrong Zheng[2], Daniel G. Meeker[3], Mark S. Smeltzer[3, 4], En Huang[1, *]

Department of Environmental and Occupational Health[1], Department of Pharmaceutical Sciences[2], Department of Microbiology and Immunology[3], Department of Orthopedic Surgery[4], University of Arkansas for Medical Sciences

4301 W. Markham Street, Little Rock, Arkansas, USA 72205

ABSTRACT

We report on the chemical synthesis and structure-activity relationship (SAR) analyses of 17 linear lipopeptide paenipeptin analogues. Three paenipeptin analogues (**7**, **12** and **17**) were more potent than the lead compound against Gram-negative and Gram-positive bacteria. Analogue **17** showed potent activity against carbapenem-resistant and polymyxin-resistant pathogens. Moreover, analogue **17** at 40 μg/mL resulted in 3 log and 2.6 log reductions of methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively, in catheter-associated biofilms *in vitro*. Analogues **9** and **16** were non-hemolytic and retained their potent *P. aeruginosa*-specific antimicrobial activity. These two analogues when used alone lacked direct activity against *Acinetobacter baumannii* and *Klebsiella pneumoniae*; however, analogue **9** and **16** at 4 μg/mL decreased the MICs of rifampicin and clarithromycin against the same pathogens from 16-32 μg/mL to nanomolar levels (sensitization factor: 2,048-8,192). Therefore, paenipeptins, alone or in combination with rifampicin or clarithromycin, are promising antibiotic candidates for the treatment of bacterial infections.

KEYWORDS: antibiotic resistance, biofilm, lipopeptide, paenipeptin, rifampicin, clarithromycin, synergism

INTRODUCTION

The continuing emergence and rapid dissemination of antibiotic-resistant pathogens are becoming a major threat to public health. Infections caused by carbapenem-resistant *Enterobacteriaceae* (CRE), which are difficult to treat and often untreatable, were recognized as one of the urgent threats among patients in medical facilities. Other serious threats caused by drug-resistant pathogens include multidrug-resistant *Acinetobacter*, multidrug-resistant *Pseudomonas aeruginosa*, drug-resistant *Campylobacter*, vancomycin-resistant *Enterococcus*, and methicillin-resistant *Staphylococcus aureus.*[1] Recently, the plasmid-mediated polymyxin-resistant gene, *mcr-1*, from Gram-negative bacteria was reported in many countries all over the world.[2] These findings implied a major breach of the last line of defense against drug-resistant pathogens. Compounding the concern, many bacterial pathogens are capable of forming biofilms, which are matrix-embedded cell aggregates and intrinsically resistant to most antibiotic treatment, on both native host tissues and indwelling medical devices.[3] Serious infections caused by antibiotic-resistant pathogens are associated with high rates of morbidity and mortality and contribute to significant economic costs. Therefore, there is an urgent need to develop novel, safe, and effective antimicrobial agents for the treatment of infections caused by drug-resistant and biofilm-forming pathogens.

Gram-negative bacteria are intrinsically resistant to large hydrophobic molecules, including many antibiotics, as a result of the permeability barrier of the outer membrane. The integrity of the outer membrane lies in the anionic lipopolysaccharides (LPS) network linked by divalent cations (Mg^{2+} and Ca^{2+}) on the cell surface.[4] Cationic peptides or lipopeptides, which have affinity to LPS, may disorganize the outer membrane and allow the entry of existing antibiotics in clinical use into the Gram-negative bacterial cells. Polymyxin B nonapeptide (PMBN),

polymyxin NAB 7601/741,[5,6] unacylated tridecaptin A1,[7] and oligo-acyl-lysyls[8] were reported to sensitize Gram-negative bacteria to antibiotics that are excluded by an intact outer membrane.

Paenipeptin C' (Figure 1) is a novel synthetic linear lipopeptide antibiotic based on a natural mixture of linear and cyclic lipopeptides produced by *Paenibacillus* sp. OSY-N. We conducted the first chemical synthesis of linear paenipeptin and observed that cyclization through the macrolactone ring is not essential for the antimicrobial activity of the paenipeptin family.[9] Other synthetic linear lipopeptides based on natural products include tridecaptin A1[10] and battacin.[11] The development of linear lipopeptide antibiotics is economically beneficial because it significantly simplifies the synthetic process and thus reduces the cost for manufacturing this family of lipopeptides. Most importantly, this allows us access to a large number of linear paenipeptin analogues using standard solid-phase peptide synthesis. Based on these findings, we used paenipeptin C' as a lead compound for developing a series of linear paenipeptin analogues. The objectives of this study were to determine structure-activity relationship (SAR) of 17 linear paenipeptin analogues in order to identify candidates with improved antibacterial activity and/or reduced toxicity.

RESULTS AND DISCUSSION

Rationale behind the choice of paenipeptin analogues

Paenipeptin analogues were designed based on SAR and the general mechanism of action of other cationic lipopeptides, which can bind to the negatively-charged lipopolysaccharides on the outer membranes of Gram-negative bacteria, followed by compromising the cytoplasmic membranes.[10,12,13] There are three key structural features in the cationic lipopeptide paenipeptin: the hydrophobic N-terminal fatty acyl chain, the positively-charged residues, and the hydrophobic amino acids. In this study, we aimed to determine the impact of the length of lipid

chain, the type and number of positively-charged amino acids, and the overall hydrophobicity on the activity of paenipeptin analogues. Peptide sequences of 17 synthetic paenipeptin analogues are summarized in Table 1. The structure variations in these analogues include: (i) varying the length of the N-terminal fatty acyl chain (hexanoyl, heptanoyl, octanoyl and decanoyl groups in analogues **1**, **3**, **8**, and **12**, respectively); (ii) replacement of the fatty acyl chain with hydrophobic acyl groups (benzoyl, benzyloxycarbonyl, and 3-cyclohexylalanyl groups in analogues **14**, **15**, and **16,** respectively); (iii) modification of the positively-charged residues (analogues **9**, **10**, **11,** and **13**); and (iv) changing the hydrophobic amino acids in paenipeptin (analogues **2**, **4**, **5**, **6**, **7,** and **17**).

***In vitro* antibacterial activity**

The antibacterial activities of 17 paenipeptin analogues were determined against 4 Gram-negative and 3 Gram-positive strains through minimum inhibitory concentration (MIC) measurement. The MIC values (μg/mL) of all paenipeptin analogues against tested bacterial species are listed in Table 2. With the increase of the fatty acid chain length from C6 to C10 in analogues **1**, **3**, **8** and **12**, the antibacterial activity increased. Analogues **1** and **3**, in which the lipid chains are shorter than C8, were significantly less active than analogue **8**, whereas analogue **12** which contains a C10 lipid tail displayed a remarkable increase in its antibacterial activity. Replacement of the lipid chain with benzoyl or benzyloxycarbonyl group (analogues **14** and **15**) diminished their antimicrobial activities, but the 3-cyclohexylalanyl substituent in analogue **16** retained its potency against *E. coli* and *P. aeruginosa* (Table 2).

The lead compound **8** consists of 3 positively charged 2,4-diaminobutyric acid (Dab) residues; replacement of all 3 Dab residues by ornithine (Orn), which possesses an additional carbon on the side chain, unexpectedly abolished the antibacterial activity of analogue **11**. Alternations of

the number of positively-charged residues had considerable effects on the antimicrobial activity. For example, altering the C-terminal Ser to Dab at position 9 in analogue **9** changed it to a narrow-spectrum antibiotic with potent activity against *P. aeruginosa*. However, adding one additional Dab at position 2 (analogue **10**) or reducing the Dab charge at position 3 (analogue **13**) resulted in almost complete loss of their antibacterial activity. Hydrophobicity changes by residue substitutions in paenipeptin analogues also influenced their antibacterial activity. Analogues **3** and **4** with a more hydrophobic residue (Ile or Phe) at position 2 were more potent than analogue **2** with a Val residue at the same position. Similarly, a replacement of Val by Leu at position 7 (analogue **5**) showed an increase in antimicrobial activity. Further increase of the hydrophobicity in analogues **6, 7,** and **17** significantly enhanced the antibacterial activity against all tested bacteria (Table 2).

Eight paenipeptin analogues showing potent antibacterial activity were chosen to determine their minimum bactericidal activity (MBC). Analogues **9** and **16** showed narrow activity against *Pseudomonas*; therefore only *P. aeruginosa* was tested for these two compounds. The MBC values (μg/mL) of selected paenipeptin analogues are listed in Table 3. Compared to the lead compound **8**, three new paenipeptin analogues (**7**, **12**, and **17**) displayed a 2-8 fold increase in their bactericidal activity against most tested bacteria (Table 3).

Time-kill assays were performed for the most effective analogue **17** against *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213 at three concentrations (8, 16 and 32 μg/ml). Analogue **17** showed concentration dependent bactericidal effect against both pathogens. *P. aeruginosa* cells were reduced by 5.1 log and 3.7 log within 2 h when treated with analogue **17** at 32 μg/ml and 16 μg/ml, respectively; no viable cells were detected at 24 h at both concentrations. Analogue **17** at 8 μg/ml resulted in 2.2 log reduction of *P. aeruginosa* cells in 2 h but significant bacterial

regrowth was observed at 24 h (Figure 2A). It took longer time for analogue **17** to achieve similar inactivation of *S. aureus* cells; analogue **17** at 32 μg/ml and 16 μg/ml inactivated 5.4 log and 3.1 log *S. aureus* cells, respectively, within 4 h. Regrowth of *S. aureus* was also observed when the cells were treated with analogue **17** at 8 μg/ml (Figure 2B).

Impact of human serum on antibacterial activity and stability

Human serum alone inhibited the growth of *P. aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 13883 (data not shown); therefore, other reference strains (*E. coli* ATCC 25922 and *S. aureus* ATCC 29213) that grew in 95% human serum were used to test the antibacterial efficacy of paenipeptin analogues. Analogues **9** and **16** showed potent activity in human serum against *E. coli*; analogue **9** also exhibited a slight increase in its activity against *S. aureus* in the presence of human serum (Table 4). However, other analogues showed varying extents of reduction in antimicrobial efficacy in the presence of human serum. For example, analogue **17** showed 8-16 times decrease in its efficacy against both *E. coli* and *S. aureus*, which corresponded to 88%-94% reduction in its activity. It is generally believed that human serum proteins can bind to certain lipopeptide antibiotics and limit their antibacterial activity. For example, the MIC of lipopeptide MX-2401 against *S. aureus* ATCC 29213 increased from 2 μg/ml in microbiological medium to 128 μg/ml in 95% mouse serum; this corresponded to 98.9% protein binding. [14] However, the degree of protein binding cannot be used to accurately predict the ultimate therapeutic performance of antibiotics. For instance, daptomyxin is a lipopeptide highly bound to plasma proteins (94%) [15], but it is an effective drug recently approved to treat infections caused by methicillin-resistant *S. aureus*.

Among eight paenipeptin analogues tested in the presence of 95% human serum, four analogues (**8**, **9**, **16** and **17**) showed relatively high efficacy against *E. coli* (Table 4), thus these four

analogues were further tested for their stability at 37°C in human serum. The residual activity after incubation in serum was quantified by determining the MIC of the treated paenipeptin analogues. Analogue **16** showed an increase in its MIC from 1-2 μg/ml to 32 μg/ml after incubation in serum for 6 h, which indicated that analogue **16** was not stable in human serum (Table 5). Interestingly, analogue **16** displayed a relative low MIC (2-4 μg/ml) in human serum against strain *E. coli* (Table 4) but lost its efficacy after an extended incubation time in serum; these data together suggested that analogue **16** may rapidly kill bacterial cells before being inactivated by serum components during the MIC test when the lipopeptide and bacterial cells coexisted in serum. Most importantly, other three tested analogues (**8**, **9** and **17**) showed little decrease in their antibacterial activity during incubation in human serum for 24 h (Table 5). Analogue **8** differs from analogue **16** in the N-terminal modifications. The N-terminal lipid chain in analogue **8** was associated with its high stability in human serum; however the hydrophobic group (3-cyclohexylalanyl) in analogue **16** did not protect it from being inactivated in serum. Analogue **16** and analogue **17** shared the same N-terminal modification, but the latter became more stable when three amino acids in positions 2, 7, and 8 were substituted by more hydrophobic residues.

Hemolytic activity

Alternation of the N-terminal lipid chain had a substantial impact on paenipeptin hemolytic activity. Among analogues **1**, **3**, **8**, and **12**, hemolytic activity against rabbit red blood cells increased as the lipid chain length increased from C6 to C10. Specifically, analogues **1** and **3** with a C6 and C7 lipid chain, respectively, displayed little hemolysis at 128 μg/mL, whereas at the same concentration, analogue **12** carrying a C10 lipid chain showed strong hemolytic activity (Table 6). Importantly, these same trends were reflected in relative antibacterial activity, which

also increased with increasing chain length. By contrast, replacing the fatty acid chain with hydrophobic groups in analogues **14**, **15**, and **16** greatly reduced their hemolytic activity. These results are in agreement with previous reports where replacing the fatty acid chain with aromatic groups reduced the toxicity of lipopeptide polymyxins.[16,17] However, as noted above, replacing the fatty acid chain with hydrophobic groups in analogues **14** and **15** also resulted in reduced antibacterial activity.

Analogues **9** and **16**, which retained their activity against *Pseudomonas,* were non-hemolytic at 128 μg/mL (Table 6), thus these two paenipeptin derivatives could be further developed as narrow spectrum anti-*Pseudomonas* agents. Among C7 paenipeptin derivatives (analogues **2**-**7**), hemolytic activities increased when more hydrophobic amino acids were introduced into the peptide chain; one exception was analogue **6**, which was more hydrophobic but much less hemolytic than analogue **5**. Analogue **17** showed at least 4 times improvement in its antibacterial activity against *A. baumannii* and *K. pneumoniae* (Table 2) over analogue **8** at the expense of a 37% increase in its hemolytic activity (Table 6).

Antibacterial activity of analogue 17 against antibiotic-resistant bacteria

Paenipeptin analogue **17** is the most active compound among 17 rationally-designed analogues. Therefore, this analogue was further evaluated for its *in vitro* efficacy against drug-resistant Gram-negative bacteria. Paenipeptin analogue **17** showed potent activity with minimum inhibitory concentrations (MIC) of 0.5-2 μg/ml against 9 carbapenem-resistant clinical isolates from FDA-CDC Antibiotic Resistance Bank, including *A. baumannii*, *Enterobacter cloacae*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa* (Table 7). In addition, analogue **17** is very effective against polymyxin-resistant *E. coli* and *K. pneumoniae* strains, including an isolate carrying the plasmid-encoded polymyxin resistance gene, *mcr-1* (Table 8).

Antibiofilm activity of analogue 17

Bacterial cells in biofilms are more tolerant than planktonic cells to antibiotic treatment, thus making it important to identify those antibiotics that have the greatest efficacy in the context of an established biofilm. Previous studies demonstrated that daptomycin and ceftaroline are more active in this context than other antibiotics tested.[18] For this reason, evaluation of analogue **17** was based on comparison to these two antibiotics. As shown in Figure 3A, analogue **17** showed activity comparable to both of these antibiotics when tested *in vitro* in the context of established catheter-associated biofilms formed by the methicillin-resistant *S. aureus* strain LAC. Specifically, treatment with analogue **17** at 40 μg/mL (5 × MIC) or 160 μg/mL (20 × MIC) resulted in 3 log and 5 log reductions, respectively, of biofilm-associated *S. aureus* cells. Moreover, at a concentration equal to 20× its MIC, analogue **17** completely cleared three of six catheters of viable bacteria (50%), whereas the positive control daptomycin only cleared one of six catheters (17%), and none were cleared by an equivalent concentration (based on MIC) of ceftaroline. Analogue **12**, which displayed the greatest activity against planktonic cells of *S. aureus* strain LAC, lacked activity against established catheter-associated biofilms (data not shown). Analogue **17** was also tested against biofilms formed by *P. aeruginosa* ATCC 27853 using the same *in vitro* catheter-associated biofilm model. Bacterial counts on catheters after treatment revealed that analogue **17** at 40× MIC (40 μg/ml) resulted in 2.6 log reduction of *P. aeruginosa* cells in established biofilms (Figure 3B).

Sensitization of Gram-negative pathogens to conventional antibiotics

Gram-negative bacteria, including *Acinetobacter* and *Klebsiella*, are intrinsically resistant to the hydrophobic antibiotic rifampicin. The MICs of rifampicin alone against *A. baumannii* ATCC 19606 and *K. pneumoniae* ATCC 13883 were 16 μg/mL (Table 9). Ten paenipeptin analogues,

which were devoid of direct antibacterial activity against *Acinetobacter* and *Klebsiella* when used alone, were investigated for a potential synergistic effect with rifampicin. Paenipeptin analogue **13** was the only tested compound that did not show synergism with rifampicin. Analogue **13** lacked antimicrobial activity and synergism with rifampicin likely due to the replacement of the positively-charged Dab with Thr at position 3 in the peptide. This substitution reduces the overall charges of the peptide and thus may decrease the interaction with LPS in Gram-negative bacteria. Therefore, analogue **13** was not able to promote the entry of rifampicin into Gram-negative pathogens. Nine analogues displayed various degrees of synergistic effects with rifampicin. When tested at 4 μg/mL, six paenipeptin analogues (**1**, **3**, **9**, **14**, **15** and **16**) decreased the MICs of rifampicin against both pathogens from 16 μg/mL to a range between < 0.00098 μg/mL and 0.0078 μg/mL, which corresponded to 2,048-8,192 times increase of the antibacterial activity of rifampicin (Table 9).

Two paenipeptin analogues (**9** and **16**) that showed promising synergism with rifampicin were selected for further test in combination with four additional conventional antibiotics with various mechanisms of action. Clarithromycin and erythromycin block protein synthesis whereas vancomycin and ampicillin inhibit cell wall biosynthesis. These four conventional antibiotics when used alone were not effective against Gram-negative pathogens (MIC≥32 μg/ml). When combined with analogues **9** and **16** at 4 μg/ml, clarithromycin showed 2,048-8,192 times increase in its activity against *A. baumannii* ATCC 19606 and *K. pneumoniae* ATCC 13883 (Table 10). In the presence of **9** and **16** at 4 μg/ml, erythromycin exhibited a moderate increase (64-512 times) against these two pathogens. Interestingly, analogues **9** and **16** potentiated the activity of vancomycin against *A. baumannii* but not *K. pneumoniae*. No synergistic effect was observed between **9** or **16** and ampicillin (Table 10).

Mechanism of action

Purified lipopolysaccharides (LPS) have a significant impact on the antimicrobial activity of paenipeptin analogue **17** against *P. aeruginosa* ATCC 27853. The addition of purified LPS at a high concentration (100 µg/ml) neutralized the bactericidal activity of **17** (Figure 4A). A similar trend was observed for analogues **7, 8**, **9** and **12** (data not shown). These results suggest that paenipeptin has a high affinity for LPS from the outer membrane of Gram-negative bacteria. Therefore, LPS on the Gram-negative cell surface is likely the initial binding target of paenipeptin. In Gram-positive bacteria, the negatively charged lipoteichoic acids (LTA) is an important component on the cell surface. As shown in Figure 4B, at 100 µg/mL, purified LTA significantly reduced the antibacterial activity of **17** against *S. aureus* ATCC 29213. LTA at 50 µg/ml showed certain inhibition on activity, but the effect was not statistically different. These results suggest that LTA in Gram-positive bacteria can serve as a docking molecule for paenipeptins, likely through electrostatic interaction. The negatively-charged LTA was reported as the initial target of several cationic peptides, including nisin and brevibacillin. [19]

Bacterial cells maintain a proton gradient across the cytoplasmic membrane where electrical potential gradient is a main component of the proton motive force. Paenipeptin analogue **17** disrupted the cytoplasmic membrane in both Gram-negative and Gram-positive bacteria. As shown in Figure 5, paenipeptin analogue **17** at ≥ 16 µg/mL depolarized the membrane potential as evidenced by the increase of fluorescence due to the release of a $DiSC_3(5)$ probe, which is only accumulated in healthy, polarized cell membranes. Moreover, analogue **17** at 32-64 µg/mL significantly released intracellular potassium ions from treated *P. aeruginosa* and *S. aureus* cells (Figure 6). Therefore, the bactericidal activity of paenipeptins can likely be attributed to the disruption and damage of the cytoplasmic membranes.

CONCLUSIONS

Through SAR studies, we have identified three paenipeptin analogues (**7**, **12** and **17**) which were more potent than the lead compound **8**. However, analogue **12** showed significant hemolysis, which suggests cytotoxicity and restricts its further development for therapeutic use. Analogue **17** showed potent activity against methicillin-resistant *S. aureus* biofilms comparable to that observed with daptomycin and ceftaroline. This new analogue also exhibited strong efficacy against established *P. aeruginosa* biofilms. Therefore, analogue **17** is a promising broad-spectrum antibiotic candidate targeting drug-resistant pathogens under both planktonic and biofilm-associated conditions. Analogue **9**, which differs from the lead compound **8** by one amino acid, was non-hemolytic and retained potent *P. aeruginosa*-specific antimicrobial activity. Several polymyxin derivatives lacking the N-terminal fatty acyl chain also showed narrow spectrum activity against *P. aeruginosa*.[20,21] One of the advantages of using such narrow spectrum antibiotics is to lower the antibiotic pressure on commensal bacteria.

Many potent large hydrophobic antibiotic drugs, including rifampicin, clarithromycin and erythromycin, are not active against Gram-negative pathogens because of the outer membrane permeability barrier. LPS in the outer membrane of Gram-negative bacteria is the major permeability barrier that excludes hydrophobic drugs.[4] Considering the urgent need for treating infections caused by multi-drug resistant Gram-negative pathogens, outer membrane permeabilizers, which promote the entry of existing antibiotics into Gram-negative pathogens, provide an alternative approach to combat antibiotic resistance.[22] Polymyxin B nonapeptide (PMBN), a polymyxin derivative lacking the fatty acid tail, was reported to sensitize *E. coli* and *Salmonella* Typhimurium to antibiotics by a factor of 30-300. However, PMBN showed similar nephrotoxicity to its parent compound, polymyxin B.[4] Polymyxin NAB7061 and polymyxin

NAB741, the second generation polymyxin derivatives with only three positive charges, reduced the MICs of rifampicin by a factor up to 2,000 when used at 4 μg/ml.[5,6] Similarly, unacylated tridecaptin A_1 reduced the MIC of rifampicin against *K. pneumoniae* strains by 256-512 fold.[7] Jammal et al.[8] reported that oligo-acyl-lysyls (C10X) at 5 μg/mL can reduce the MICs of rifampicin against Gram-negative bacteria from 8-32 μg/mL to 0.0044-0.031 μg/mL (sensitization factor: 258-4,000). Among the 17 paenipeptin analogues in this study, 10 compounds, which showed little hemolytic activity and were devoid of direct activity against *A. baumannii* and *K. pneumoniae*, were tested in combination with rifampicin against these two pathogens. When tested at 4 μg/ml, six paenipeptin analogues (**1**, **3**, **9**, **14**, **15** and **16**) decreased the MIC of rifampicin against *A. baumannii* and *K. pneumoniae* from 16 μg/mL (19.42 μM) to nanomolar and subnanomolar levels (sensitization factor: 2,048-8,192). A similar high synergy was also observed between analogue **9** or **16** and a protein synthesis inhibitor, clarithromycin. Therefore, paenipeptins can potentiate different classes of antibiotics with varying mode of action. These results suggest that a combined treatment of paenipeptin analogues with rifampicin or clarithromycin is a very attractive option to treat *A. baumannii* and *K. pneumoniae* infections.

EXPERIMENTAL SECTION

Synthesis and characterization of paenipeptin analogues. Seventeen paenipeptin analogues were synthesized through a commercial custom peptide service (Genscript Inc., Piscataway, NJ). Solid-phase peptide synthesis was carried out using Fmoc chemistry on rink amide resin. The resin was pre-swollen in DMF for 1 h before usage. The amidation reaction was achieved by the addition of corresponding amino acid, HOBT and DIC followed by being rocked at room temperature for 1 h. Fmoc protecting group was removed by the treatment of 20% piperidine (v/v) in DMF for 1 h. Between deprotection and coupling, the solid phase peptide synthesis vessel was

drained under N_2 pressure and washed with DMF for 5 times. A small portion of resin was cleaved and analyzed by HPLC to confirm the conversion. Finally, the resin was treated with a mixture of TFA/TIPS/H_2O (18:1:1, v/v/v) and gently shaken for 2 h. The cleavage solution was filtered and concentrated in vacuo. The crude peptides were purified using preparative-scale C_{18}-RP-HPLC. Synthetic lipopeptides were purified by HPLC to homogeneity (≥ 95% purity) and characterized by high resolution mass spectrometry. The mass spectra (MS) were recorded using Agilent 6210 Time-of-Flight (TOF) LC Mass Spectrometer (Agilent Technologies, Santa Clara, CA) at University of California at Riverside. ^{1}H NMR spectra of each paenipeptin analogue were recorded in D_2O on an Agilent 400-MR DD2 Spectrometer (400 MHz) using Tetramethylsilane (TMS) as internal standard. Chemical shifts were reported as δ (ppm) and spin-spin coupling constants as J (Hz) values. Purity, HRMS and ^{1}H NMR data were presented in the supplemental materials.

Antimicrobial susceptibility tests. The minimum inhibitory concentration (MIC) of paenipeptin analogues was determined using the broth microdilution method.[23] Briefly, seven reference strains, *Acinetobacter baumannii* ATCC 19606, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecium* ATCC 19434, *Staphylococcus aureus* ATCC 29213 and methicillin-resistant *S. aureus* ATCC 43300 were used for the MIC tests. Paenipeptin analogues were two-fold diluted in tryptic soy broth (TSB; Becton Dickinson) and mixed with an equal volume of bacterial suspension in TSB (approximately 1.5×10^5 CFU/mL) in a clear UV-sterilized 96-well microtiter plate (NBS, Corning Inc., Corning, NY). The total volume was 100 µL and the final paenipeptin concentrations ranged from 0.5-32 µg/mL. The microtiter plate was incubated at 37 °C for 18-20 h. MIC referred to the lowest concentration of paenipeptins which resulted in no visible growth

of bacterial cells after incubation. In addition to above reference strains, the susceptibility of nine carbapenem-resistant isolates and six polymyxin-resistant strains (Tables 7 and 8), including clinical isolates from FDA-CDC antibiotic resistance bank, were tested for the selected paenipeptin analogue **17**. There were at least three independent experiments with one replicate in each experiment.

Minimum bactericidal concentration (MBC) of paenipeptin analogues was determined at the end point of MIC tests by sub-culturing an aliquot of 50 µL cell suspension from the 96-well microtiter plate. The surviving cells from each antimicrobial concentration with no visible growth were enumerated using tryptic soy agar (TSA). MBC is defined as the lowest concentration of the antimicrobial agent that leads to at least 99.9% reduction of the initial inoculum in the broth. There were three independent experiments with one replicate in each experiment.

Time-kill kinetics of the selected analogue **17** (0, 8, 16 and 32 µg/ml) were determined using two reference strains, *P. aeruginosa* ATCC 27853 and S. *aureus* ATCC 29213. The surviving cells after antimicrobial treatment were enumerated at 0, 2, 4, 6 and 24 h using TSA. There were three independent experiments with one replicate in each experiment.

Impact of human serum on antibacterial activity and stability. The impact of human serum on the antibacterial activity of selected paenipeptin analogues was determined in the presence of human serum (MP Biomedicals, Solon, OH). The procedure was similar to the MIC determination as described above but the microbiological medium was replaced by 95% human serum. Paenipeptin analogues were two-fold diluted in human serum and mixed with an equal amount of diluted *E. coli* ATCC 25922 or *S. aureus* ATCC 29213 cells (approximately 1.5×10^5

CFU/mL) in human serum for MIC determination. The MIC in the presence of serum was recorded and compared to the value without serum.

To determine the stability of peptides in human serum, paenipeptin analogues were added into 100% human serum to a final concentration of 64 μg/ml. The mixtures were incubated at 37 °C and samples were withdrawn at 0, 6, 12 and 24 h. The treated paenipeptin analogues were two-fold diluted in human serum and mixed with an equal volume of *E. coli* ATCC 25922 cells in TSB (approximately 1.5×10^5 CFU/mL) for MIC determination. The residual antibacterial activity after incubation in human serum was compared to the non-treated control.

Sensitization of Gram-negative pathogens to rifampicin. Synergism of paenipeptin analogues with rifampicin against two pathogens, *A. baumannii* ATCC 19606 and *K. pneumoniae* ATCC 13883, was determined using a checkerboard method similar to the MIC determination.[6] The MICs of rifampicin (Sigma, St. Louis, MO) were determined in the presence of 0, 2, and 4 μg/mL of 10 selected paenipeptin analogues, which lack direct antimicrobial activity against *A. baumannii* ATCC 19606 and *K. pneumoniae* ATCC 13883 (MICs ≥ 16-32 μg/mL; Table 2). A sensitization factor is defined as the ratio of the rifampicin MICs in the absence of paenipeptins to that in the presence of 4 μg/mL of paenipeptins. Similarly, two selected paenipeptin analogues (**9** and **16**) were further tested at 4 μg/mL in combination with four additional conventional antibiotics, including ampicillin (Sigma), clarithromycin (Sigma), erythromycin (Sigma), and vancomycin (Sigma). There were at least three independent experiments with one replicate in each experiment.

Determination of hemolytic activity. Hemolytic activities of 17 paenipeptin analogues were evaluated using defibrinated rabbit blood in a 96-well plate as described previously.[10] A nonionic

surfactant Triton X-100, which is capable of lysing red blood cells, was used at 0.1% as a positive control. Briefly, rabbit blood (Hardy Diagnostics, Santa Maria, CA) was diluted with phosphate buffered saline (PBS; pH 7.2) at a 1:19 ratio (v/v) and free hemoglobin was removed by centrifugation four times at 1,000 × *g* at 4 °C for 5 min. Aliquots (50 µL) of washed red blood cells were incubated with 150 µL two-fold dilutions of paenipeptin analogues at final concentrations of 16-128 µg/mL at 37°C for 30 min in a microtiter plate (NBS, Corning Inc.). After incubation, treated red blood cells were gently mixed by repeated pipetting. Aliquots (20 µL) of the cell suspension were mixed with 200 µL PBS in a new 96-well plate and centrifuged at 2,204 × *g* for 10 min. The supernatant was transferred to a new 96-well plate for absorbance measurement at 415 nm using a Cell Imaging Multimode Reader (Cytation 3, BioTek; Winooski, VT) and percentage hemolysis of paenipeptin analogues was calculated relative to Triton X-100. There were at least three independent experiments with one replicate in each experiment.

Determination of efficacy against catheter-associated biofilms *in vitro*. The effect of paenipeptin analogue **17** on established *S. aureus* or *P. aeruginosa* catheter-associated biofilms was determined *in vitro* as described previously with minor modifications.[18] Briefly, 1-cm segments (n=6) of fluorinated ethylene propylene catheters (14 gauge; Introcan safety catheter; B. Braun, Bethlehem, PA), which were pre-coated with human plasma overnight, were placed into 2 mL biofilm medium (TSB supplemented with 0.5% glucose and 3.0% NaCl)[24] in wells of a 24-well microtiter plate (ultra-low attachment surface; Corning Inc.). Each well was inoculated with the methicillin-resistant *S. aureus* strain LAC or *P. aeruginosa* ATCC 27853 to an OD_{600nm} of 0.05. After incubation at 37 °C for 24 h, catheters with established biofilm were transferred to a freshly made biofilm medium without or with paenipeptin analogue **17** (5×, 10×, and 20× its MIC for the *S. aureus* LAC strain; 10×, 20×, and 40×, and 80× its MIC for *P. aeruginosa* ATCC

27853). The MIC of analogue **17** against LAC and *P. aeruginosa* ATCC 27853 were 8 μg/mL and 1 μg/ml, respectively. Catheters were removed after 24 and 48 h, rinsed with sterile PBS, and transferred to fresh biofilm medium containing the same concentration of antibiotics. After 72 h of treatment, catheters were rinsed with PBS, followed by sonication for recovering adherent bacteria from biofilms. Viable *S. aureus* or *P. aeruginosa* cells were enumerated using tryptic soy agar plates. Ceftaroline and daptomycin were used as positive controls at 20× the MIC for each antibiotic against LAC.[18] Polymyxin B was used as a positive control at 20× its MIC (10 μg/ml) against *P. aeruginosa* ATCC 27853. There were six independent experiments with one replicate in each experiment.

Interaction of paenipeptin analogues with lipopolysaccharides and lipoteichoic acid. To identify the initial binding target of paenipeptin on the cell surface, lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria or lipoteichoic acid (LTA) from Gram-positive bacteria were investigated for their impact on bactericidal activities of paenipeptin analogue **17**. *P. aeruginosa* ATCC 27853 culture was diluted with tryptic soy broth to contain approximately 10^6 CFU/mL. LPS from *E. coli* O111:B4 (Sigma) was added to the cell suspension at a final concentration of 10, 25, 50, or 100 μg/mL. This was followed by adding paenipeptin analogue **17** to a final concentration of 16 μg/mL. The mixtures were incubated at 37 °C with agitation at 200 rpm for 60 min. Surviving cells were quantified by spread-plating on tryptic soy agar.[12] There were three independent experiments with one replicate in each experiment. Similarly, *S. aureus* ATCC 29213 was diluted to ~10^6 CFU/mL and mixed with LTA (Sigma) isolated from *S. aureus* to a final concentration of 10, 25, 50, or 100 μg/mL. After adding **17** to a final concentration of 32 μg/mL and incubation at 37 °C with agitation at 200 rpm

for 60 min, surviving *S. aureus* cells were enumerated on tryptic soy agar. There were four independent experiments with one replicate in each experiment.

Cytoplasmic membrane integrity assay. The disturbance of membrane potential after paenipeptin treatment was determined using a fluorescent probe, 3,3'-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$; Invitrogen]. $DiSC_3(5)$ is membrane potential-sensitive dye, which accumulates in polarized cytoplasmic membranes and becomes self-quenched. An overnight culture of *P. aeruginosa* ATCC 27853 or *S. aureus* ATCC 29213 was diluted 1/100 in TSB and grown at 37 °C with agitation at 200 rpm for ~5 h. After incubation, bacterial cells were harvested by centrifugation at 3,660 × *g* at 4 °C for 10 min and washed twice using 5 mM HEPES buffer (pH 7.2, Sigma) supplemented with 5 mM glucose (buffer A). Cells of S. *aureus* were resuspended in buffer A. However, *P. aeruginosa* cells were resuspended in buffer B (buffer A supplemented with 0.2 mM EDTA), which can promote the uptake of the $DiSC_3(5)$ probe by Gram-negative bacteria. $DiSC_3(5)$ was added to the cell suspensions at a final concentration of 0.5 μM, followed by incubation for 15 min at room temperature. After incubation, KCl was added to cell suspensions at a final concentration of 100 mM. Aliquots (90 μL) of the cell suspension with integrated $DiSC_3(5)$ were added to wells of a black NBS microplate (Corning Inc.). This was followed by adding 10 μL of paenipeptin analogue **17** at a final concentration of 8-64 μg/mL. The increase of fluorescence signal due to membrane depolarization and release of the $DiSC_3(5)$ probe from bacterial cells was recorded using a Cell Imaging Multimode Reader (Cytation 3, BioTek) at an excitation of 622 nm and an emission of 670 nm.[12] There were three independent experiments with one replicate in each experiment.

Potassium ion release assay. Potassium ions leaked from paenipeptin treated bacterial cells were measured using a K^+-sensitive probe (PBFI; Invitrogen), which is impermeable to healthy

bacterial cells. Cells suspensions of *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853 were prepared in buffer A using the same procedures aforementioned in the cytoplasmic membrane integrity assay. Aliquots (90 μL) of bacterial cells were dispensed to wells of a black NBS microplate. The PBFI K^+-sensitive probe was added to the cell suspension at a final concentration of 2 μM. This was followed by adding 10 μL of paenipeptin analogue **17** at a final concentration of 8-64 μg/mL. A change in fluorescence corresponding to potassium ion concentration in the buffer was recorded using a Cell Imaging Multimode Reader (Cytation 3, BioTek) at an excitation wavelength of 346 nm and an emission wavelength of 505 nm.[12] There were three independent experiments with one replicate in each experiment.

Statistical analysis. For bacterial inactivation assays, the surviving cell counts at the terminus of experiments were analyzed. For fluorescence measurements, the changes of fluorescence strength before and after adding analogue **17** were analyzed. All data were subjected to analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) tests using SPSS Statistics (version 24; SPSS, Inc., Chicago, IL, USA).

ASSOCIATED CONTENT

Supporting information

Characterization of peptide analogues: Purity, HRMS and ^{1}H NMR data of all paenipeptin analogues

AUTHOR INFORMATION

Corresponding Author

*Phone: 501-526-6627. Fax: 501-526-6931. E-mail: ehuang@uams.edu

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

A. baumannii, *Acinetobacter baumannii*; CRE, carbapenem-resistant *Enterobacteriaceae*; CFU, colony forming unit; Dab, 2,4-diaminobutyric acid; DMF, N,N-dimethylformamide; DIC, N,N'-Diisopropylcarbodiimide; $DiSC_3(5)$, 3,3'-Dipropylthiadicarbocyanine Iodide; *E. faecium*, *Enterococcus faecium*; *E. coli*, *Escherichia coli*; Fmoc, fluorenylmethyloxycarbonyl; HOBT, hydroxybenzotriazole; *K. pneumoniae*, *Klebsiella pneumoniae*; LPS, lipopolysaccharides; LTA, lipoteichoic acids; MBC, minimum bactericidal activity; MIC, minimum inhibitory concentration; Orn, ornithine; *P. aeruginosa*, *Pseudomonas aeruginosa*; PMBN, polymyxin B nonapeptide; RP-HPLC, reverse-phase high pressure liquid chromatography; SAR, structure-activity relationship; SPPS, solid-phase peptide synthesis; *S. aureus*, *Staphylococcus aureus*; TFA, trifluoroacetic acid; TIPS, triisopropylsilane

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Table 1. Lipopeptide sequence of 17 paenipeptin analogues

Analogue	Names		Peptide sequences
1	C6-Pat	(R1=Hexanoyl)	**R1**-Dab[a]-Ile- Dab-DPhe-Leu-Dab-DVal-Leu-Ser-NH_2
2	C7Val2-Pat	(R2=Heptanoyl)	**R2**-Dab- **Val**- Dab-DPhe-Leu-Dab-DVal-Leu-Ser-NH_2
3	C7-Pat	(R2=Heptanoyl)	**R2**-Dab- Ile- Dab-DPhe-Leu-Dab-DVal-Leu-Ser-NH_2
4	C7Phe2-Pat	(R2=Heptanoyl)	**R2**-Dab- **Phe**-Dab-DPhe-Leu-Dab-DVal-Leu-Ser-NH_2
5	C7DLeu7-Pat	(R2=Heptanoyl)	**R2**-Dab- Ile- Dab-DPhe-Leu-Dab-**DLeu**-Leu-Ser-NH_2
6	C7Phe2DLeu7-Pat	(R2=Heptanoyl)	**R2**-Dab- **Phe**-Dab-DPhe-Leu-Dab-**DLeu**-Leu-Ser-NH_2
7	C7Phe2DLeu7Phe8-Pat	(R2=Heptanoyl)	**R2**-Dab- **Phe**-Dab-DPhe-Leu-Dab-**DLeu**-**Phe**-Ser-NH_2
8	C8-Pat[b]	(R3=Octanoyl)	R3-Dab- Ile- Dab-DPhe-Leu-Dab-DVal-Leu-Ser-NH_2
9	Dab9-Pat	(R3=Octanoyl)	R3-Dab- Ile- Dab-DPhe-Leu-Dab-DVal-Leu-**Dab**-NH_2
10	Dab2,9-Pat	(R3=Octanoyl)	R3-Dab-**Dab**-Dab-DPhe-Leu-Dab-DVal-Leu-**Dab**-NH_2
11	Orn-Pat	(R3=Octanoyl)	R3-**Orn**[c]-Ile- **Orn**-DPhe-Leu-**Orn**-DVal-Leu-Ser-NH_2
12	C10-Pat	(R4=Decanoyl)	**R4**-Dab- Ile- Dab-DPhe-Leu-Dab-DVal-Leu-Ser-NH_2
13	C10Thr3Leu4-Pat	(R4=Decanoyl)	**R4**-Dab- Ile- **Thr**-**DLeu**-Leu-Dab-DVal-Leu-Ser-NH_2
14	Benzoyl-Pat	(R5=Benzoyl)	**R5**-Dab- Ile- Dab-DPhe-Leu-Dab-DVal-Leu-Ser-NH_2
15	Cbz-Pat	(R6=Benzyloxycarbonyl)	**R6**-Dab- Ile- Dab-DPhe-Leu-Dab-DVal-Leu-Ser-NH_2
16	Cha-Pat	(R7=3-cyclohexylalanyl)	**R7**-Dab- Ile- Dab-DPhe-Leu-Dab-DVal-Leu-Ser-NH_2
17	ChaPhe2Leu7Phe8-Pat	(R7=3-cyclohexylalanyl)	**R7**-Dab- **Phe**-Dab-DPhe-Leu-Dab-**DLeu**-**Phe**-Ser-NH_2

[a]Dab: 2,4-diaminobutyric acid;

[b]C8-Pat: lead compound; substituted N-terminal groups and amino acid residues in other analogues are in bold;

[c]Orn: ornithine

Table 2. Comparison of minimum inhibitory concentration (MIC) of 17 synthetic paenipeptins analogues

Analogue	MIC (μg/ml)						
	A. baumannii ATCC 19606	*E. coli* ATCC 25922	*K. pneumoniae* ATCC 13883	*P. aeruginosa* ATCC 27853	*E. faecium* ATCC 19434	*S. aureus* ATCC 29213	*S. aureus* ATCC 43300
1	>32	8-16	≥32	8-16	>32	>32	>32
2	>32	16	>32	16	>32	>32	>32
3	>32	4	32	4	>32	>32	>32
4	32	1-4	8	2-4	>32	≥32	32
5	8-16	1-4	4	2-4	8-16	16-32	16
6	8	1-4	4	2-4	16	16-32	16-32
7	2-4	0.5-2	1	1-2	8	8	8
8	16	2-4	8-16	2	16	8-16	8-16
9	≥32	4-16	>32	2-4	32	>32	32
10	>32	>32	>32	16-32	>32	≥32	>32
11	32	32	>32	16	>32	>32	32
12	0.5-2	2-8	2-4	1-2	2	2-4	2-4
13	>32	>32	>32	>32	≥32	>32	>32
14	>32	16-32	>32	8-32	>32	>32	>32
15	16	8-16	>32	16	>32	>32	>32
16	≥32	4-8	32	2-8	>32	>32	32
17	2-4	0.5-1	2	0.5-1	8	2-4	4

Table 3. Comparison of minimum bactericidal concentration (MBC) of 8 selected synthetic paenipeptin analogues

Analogue	MBC (μg/ml)						
	A. baumannii ATCC 19606	*E. coli* ATCC 25922	*K. pneumoniae* ATCC 13883	*P. aeruginosa* ATCC 27853	*E. faecium* ATCC 19434	*S. aureus* ATCC 29213	*S. aureus* ATCC 43300
5	16	16	8	8-16	16	16-32	16-32
6	8	32	8	8-16	32	16-32	32
7	4	8-16	2	4	16	8-32	8-16
8	16-32	16	8-16	8-16	16-32	16	8-32
9	ND[a]	ND	ND	16	ND	ND	ND
12	0.5-2	8	4-8	2-4	2-4	4-8	2-4
16	ND	ND	ND	16-32	ND	ND	ND
17	4-8	8-16	4	2-4	16	8	8

[a] ND: not determined because of relatively high minimum inhibitory concentration

Table 4. Impact of human serum on antimicrobial activity of selected paenipeptin analogues

Analogue	Minimum inhibitory concentration (μg/ml)			
	Escherichia coli ATCC 25922		*Staphylococcus aureus* ATCC 29213	
	95% serum	TSB broth	95% serum	TSB broth
5	16	1-4	32	16-32
6	32	1-4	64	16-32
7	32	0.5-2	32-64	8
8	8	2-4	16-32	8-16
9	2-4	4-16	16	>32
12	32	2-8	16	2-4
16	2	4-8	>64	>32
17	8	0.5-1	32	2-4

Table 5. Stability of selected paenipeptin analogues in human serum at 37 °C[a]

Analogue	Minimum inhibitory concentration (μg/ml)			
	0 h	6 h	12 h	24 h
8	8	8	8	8
9	1-2	2	1-2	2
16	1-2	32	>32	>32
17	4-8	4-8	4-8	8

[a] MIC was determined against *Escherichia coli* ATCC 25922

Table 6. Comparison of hemolytic activity[a] of 17 paenipeptin analogues against rabbit red blood cells

	Hemolysis (%)			
Analogue	128 μg/mL	64 μg/mL	32 μg/mL	16 μg/mL
1	0.61±0.25	0.08±0.85	0.41±0.58	0.48±0.64
2	0.95±0.45	0.79±0.63	0.32±0.74	-0.17±0.19
3	1.77±0.81	1.18±0.68	0.78±0.30	0.18±0.54
4	1.28±1.65	0.60±1.35	0.49±1.22	0.28±1.11
5	26.5±4.45	4.86±1.05	1.39±0.55	0.96±0.59
6	8.34±1.80	2.03±0.44	0.39±0.30	0.14±0.20
7	64.2±3.28	18.8±2.44	5.10±1.26	0.99±0.31
8	25.3±4.15	6.18±2.48	1.91±0.25	0.93±0.45
9	2.25±2.18	0.73±0.50	0.80±0.68	0.52±0.58
10	0.80±0.40	0.41±0.53	0.38±0.62	0.37±0.62
11	1.12±0.47	0.69±0.64	0.44±0.30	0.86±0.72
12	93.9±7.57	73.4±7.59	37.2±8.62	14.3±4.25
13	24.9±2.71	10.0±1.03	5.06±1.14	1.25±0.71
14	0.70±0.34	1.14±1.05	0.71±0.82	0.22±0.17
15	0.65±0.29	0.54±0.70	0.41±0.67	0.77±0.15
16	1.63±0.40	0.65±0.62	0.33±0.83	0.55±0.75
17	34.6±0.91	11.02±0.99	2.46±0.34	0.60±0.18

[a] Percent hemolysis was calculated relative to the positive control, Triton X-100.

Table 7. Minimum inhibitory concentration (μg/mL) of analogue **17** against carbapenem-resistant pathogens from FDA-CDC antibiotic-resistance (AR) bank

FDA-CDC AR Bank #	Strains	Known resistance [a]	Analogue **17** (μg/ml)
063	*Acinetobacter baumannii*	OXA-23, 24/40	0.5
083	*A. baumannii*	OXA-23, NDM	2
038	*Enterobacter cloacae*	NDM	0.5
053	*E. cloacae*	KPC-3, TEM-1	0.5
048	*Escherichia coli*	NDM	0.5
061	*E. coli*	KPC-3, TEM-1	0.5-1
068	*Klebsiella pneumoniae*	NDM, OXA-181	0.5-2
097	*K. pneumoniae*	KPC	1-2
064	*Pseudomonas aeruginosa*	SPM	2

[a] Production of various types of beta-lactamase

Table 8. Minimum inhibitory concentration (μg/mL) of analogue **17** against polymyxin-resistant strains

Strains	Analogue **17** (μg/ml)	Polymyxin B (μg/ml)
Escherichia coli AR 0494 [a]	0.5	2-8
E. coli UAMS-ECPR1 [b]	<0.5	32
E. coli UAMS-ECPR2 [b]	<0.5	32
Klebsiella pneumoniae AR 0109 [a]	4-8	16-32
K. pneumoniae UAMS-KPPR1 [c]	2	>32
K. pneumoniae UAMS-KPPR3 [c]	2	>32

[a] Bacterial strains were obtained from FDA-CDC antibiotic-resistance bank, *Escherichia coli* AR 0494 carries the plasmid-encoded polymyxin resistance gene, *mcr-1*; [b] strains are the derivatives of *E. coli* ATCC 25922; [c] strains are the derivatives of *K. pneumoniae* ATCC 13883

Table 9. Minimum inhibitory concentration (MIC) of rifampicin without or with paenipeptin analogues

MIC of rifampicin (μg/ml) at the following concentrations of paenipeptin analogues								
	Acinetobacter baumannii ATCC 19606				*Klebsiella pneumoniae* ATCC 13883			
Analogue	0 μg/mL	2 μg/mL	4 μg/mL	Sensitization factor [a]	0 μg/mL	2 μg/mL	4 μg/mL	Sensitization factor
1	16	0.0078-0.0156	0.00195-0.0039	4,096-8,192	16	0.0039	0.00195	8,192
2	16	0.125	0.0313-0.0625	256-512	16	0.0156	0.0039	4,096
3	16	0.0078	0.0039	4,096	16	0.0039	< 0.00098-0.00195	≥8,192
9	16	0.0019	0.00195	8,192	16	0.0019-0.0039	< 0.00098-0.00195	≥8,192
10	16	0.03125-0.0625	0.0313-0.0625	256-512	16	0.0625-0.125	0.0625	256
11	16	0.0625	0.0156-0.0313	512-1,024	16	0.0313-0.0625	0.0078-0.0156	1,024-2,048
13	16	>0.125	>0.125	<128	16	> 0.125	>2	<8
14	16	0.03125	0.0039-0.0078	2,048-4,096	16	0.0078	0.00195-0.0039	4,096-8,192
15	16	0.0019-0.0039	<0.00098-0.00195	≥8,192	16	0.0039	0.00195-0.0039	4,096-8,192
16	16	0.0039-0.0078	0.0039	4,096	16	0.0039	< 0.00098-0.00195	≥8,192

[a] Sensitization factor: the ratio of the MIC in the absence of paenipeptins to that in the presence of 4 μg/mL of paenipeptins.

Table 10. Minimum inhibitory concentration (MIC) of ampicillin, clarithromycin, erythromycin and vancomycin with or without paenipeptin analogues **9** and **16**

MIC (μg/ml) of conventional antibiotics in the presence of 0 or 4 μg/ml paenipeptin analogues							
		Acinetobacter baumannii ATCC 19606			*Klebsiella pneumoniae* ATCC 13883		
Analogue		0 μg/ml	4 μg/ml	Sensitization factor [a]	0 μg/ml	4 μg/ml	Sensitization factor
9	Ampicillin	>32	1-2	16-32	32	32	1
	Clarithromycin	32	0.0039-0.0078	4,096-8,192	32	0.0156	2048
	Erythromycin	32	0.125-0.25	128-256	>32	0.0625	512
	Vancomycin	>32	0.25-0.5	64-128	>32	16	2
16	Ampicillin	>32	8-16	2-4	32	32	1
	Clarithromycin	32	0.0156	2048	32	0.0078-0.0156	2048-4096
	Erythromycin	32	0.5	64	>32	0.0625	512
	Vancomycin	>32	0.5-1	32-64	>32	>32	1

[a] Sensitization factor: the ratio of the MIC in the absence of paenipeptins to that in the presence of 4 μg/mL of paenipeptins.

Figure legends

Figure 1. Chemical structure of paenipeptin C' (analogue **8**)

Figure 2. Time-kill curves of pathogens with exposure to analogue **17** at 0-32 µg/mL**. (**A) *Pseudomonas aeruginosa* ATCC 27853; (B) *Staphylococcus aureus* ATCC 29213. Values are expressed as means (number of experiments, 3), and error bars represent standard deviations.

Figure 3. Relative activity of antibiotics against established biofilms on catheters *in vitro*. (A) Paenipeptin analogue **17**, ceftaroline (Cef), and daptomycin (Dap) against a methicillin-resistant *Staphylococcus aureus* strain LAC. Activity was determined at concentrations corresponding to 5×, 10×, or 20× the concentration corresponding to the MIC for each antibiotic against strain LAC. (B) Analogue **17** and polymyxin B (PMB) against *Pseudomonas aeruginosa* ATCC 27853. Activity was determined at concentrations corresponding to 10×, 20×, 40×, or 80× the concentration corresponding to the MIC for each antibiotic against *P. aeruginosa* ATCC 27853. Results were assessed after 72 h of antibiotic exposure. Values are expressed as means (number of experiments, 6). Means with different letters are significantly different between groups ($p < 0.05$).

Figure 4. Impact of lipopolysaccharides (LPS) and lipoteichoic acid (LTA) on antimicrobial activity of paenipeptin analogue **17**. (A) *Pseudomonas aeruginosa* ATCC 27853, analogue **17** at 16 µg/mL; (B) *Staphylococcus aureus* ATCC 29213, analogue **17** at 32 µg/mL. Values are expressed as means (number of experiments, at least 3), and error bars represent standard deviations. Means with different letters are significantly different between groups ($p < 0.05$).

Figure 5. Changes in bacterial membrane potential in the presence of paenipeptin analogue **17**. (A) *Pseudomonas aeruginosa* ATCC 27853; (B) *Staphylococcus aureus* ATCC 29213. Values

are expressed as means (number of experiments, 3), and error bars represent standard deviations. Means with different letters are significantly different between groups ($p < 0.05$).

Figure 6. Release of intracellular potassium ions from bacterial cells in the presence of paenipeptin analogue **17**. (A) *Pseudomonas aeruginosa* ATCC 27853; (B) *Staphylococcus aureus* ATCC 29213. Values are expressed as means (number of experiments, 3), and error bars represent standard deviations. Means with different letters are significantly different between groups ($p < 0.05$).

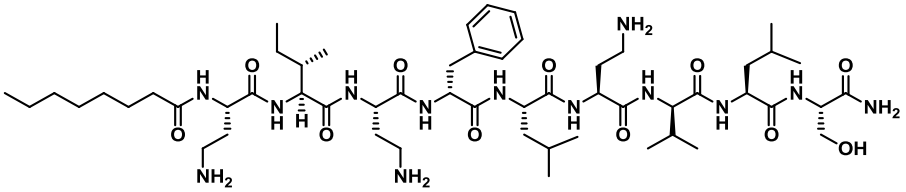
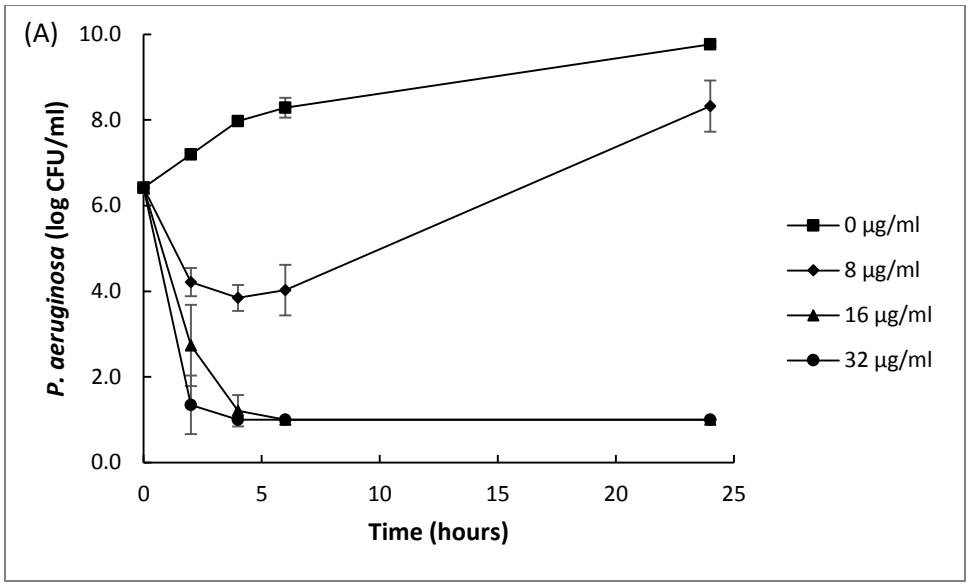


Figure 1.


(A)
10.0
8.0
6.0
4.0
2.0
0.0
P. aeruginosa (log CFU/ml)
0
5
10
15
20
25
Time (hours)
0 µg/ml
8 µg/ml
16 µg/ml
32 µg/ml

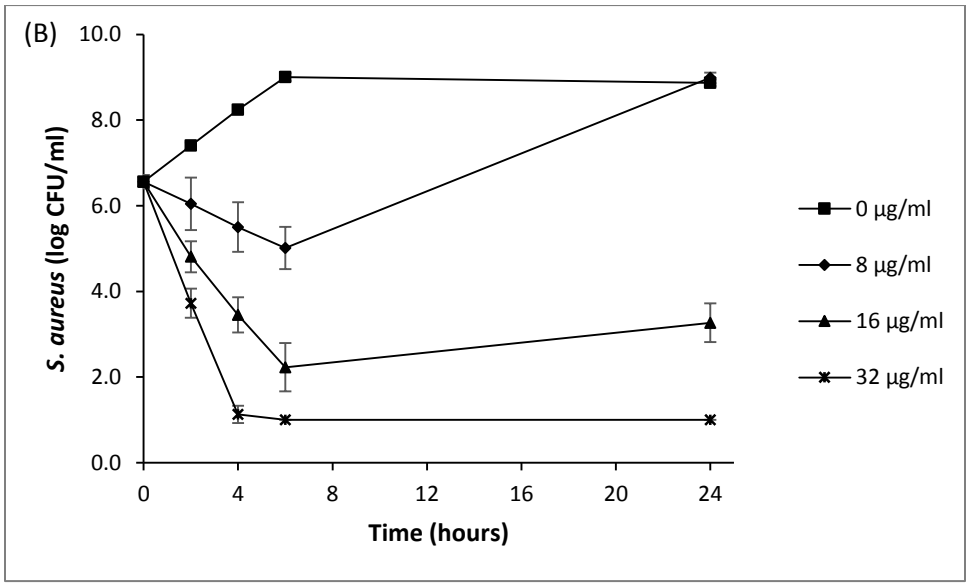

(B)
10.0
8.0
6.0
4.0
2.0
0.0
S. aureus (log CFU/ml)
0
4
8
12
16
20
24
Time (hours)
0 µg/ml
8 µg/ml
16 µg/ml
32 µg/ml

Figure 2.

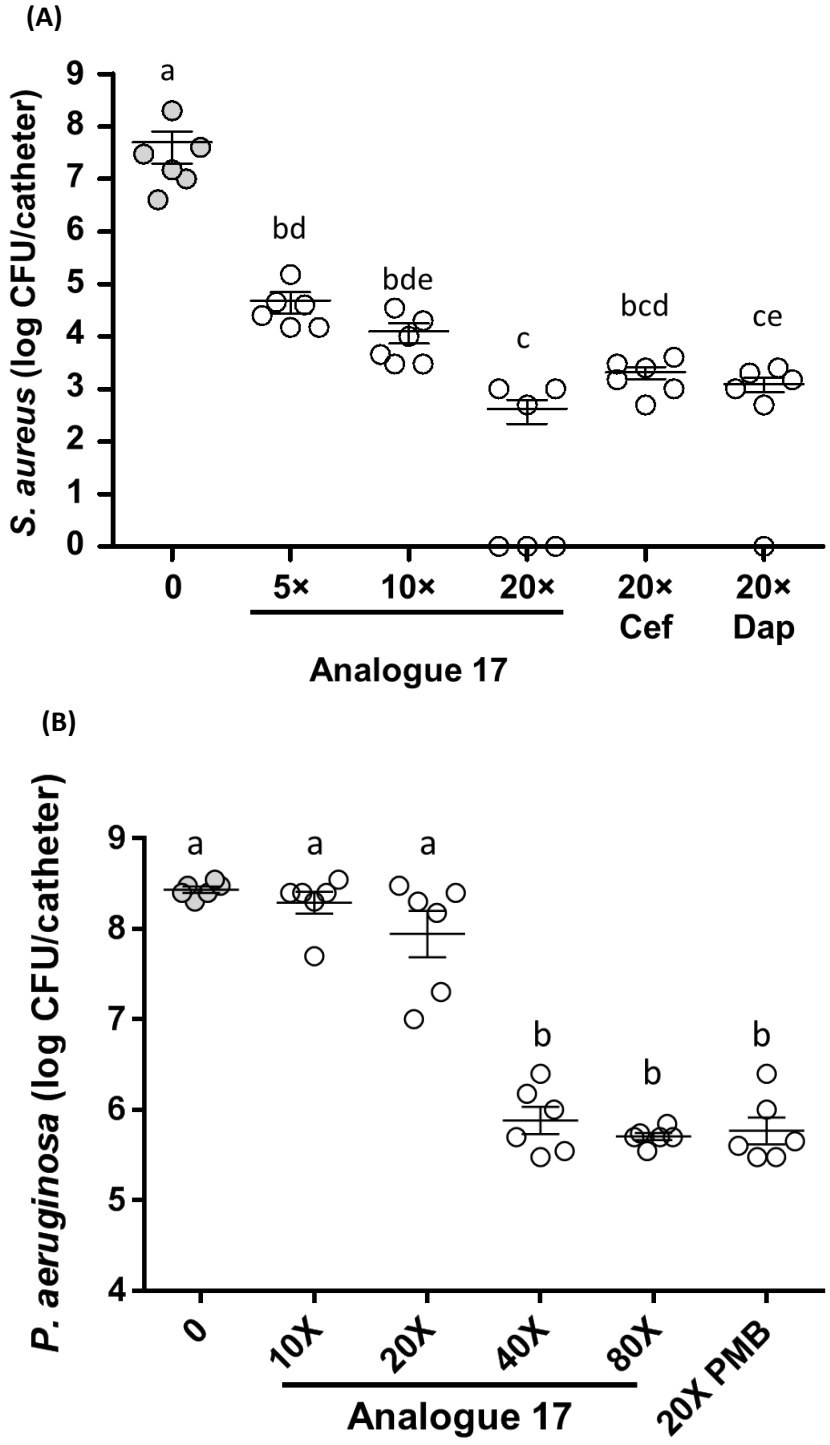

(A)
S. aureus (log CFU/catheter)
9
8
7
6
5
4
3
2
1
0
a
bd
bde
c
bcd
ce
0
5×
10×
20×
20×
Cef
20×
Dap
Analogue 17
(B)
P. aeruginosa (log CFU/catheter)
9
8
7
6
5
4
a
a
a
b
b
b
0
10X
20X
40X
80X
20X PMB
Analogue 17

Figure 3.

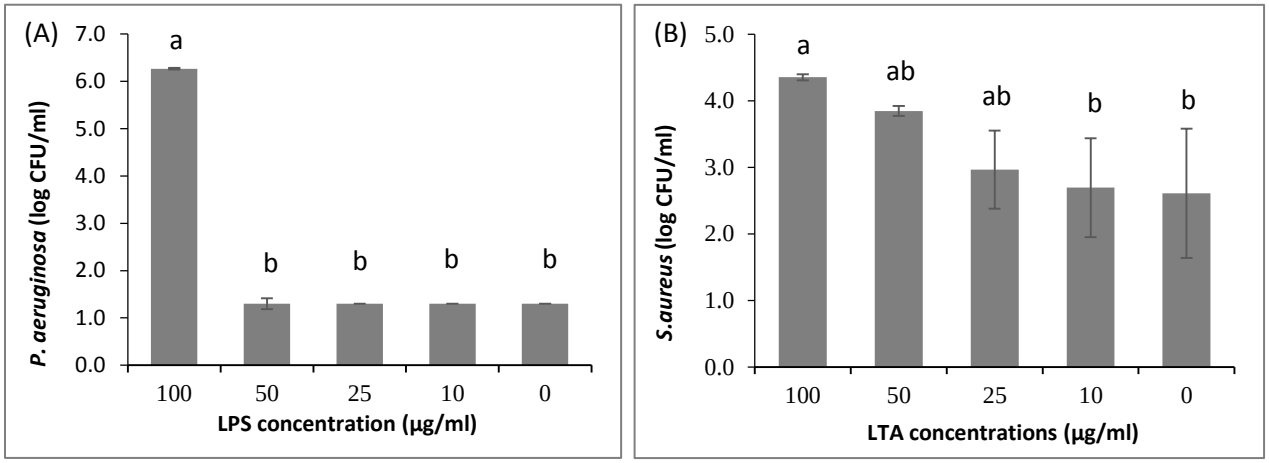

(A)
P. aeruginosa (log CFU/ml)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
a
b
b
b
b
100
50
25
10
0
LPS concentration (µg/ml)
(B)
S.aureus (log CFU/ml)
5.0
4.0
3.0
2.0
1.0
0.0
a
ab
ab
b
b
100
50
25
10
0
LTA concentrations (µg/ml)

Figure 4.

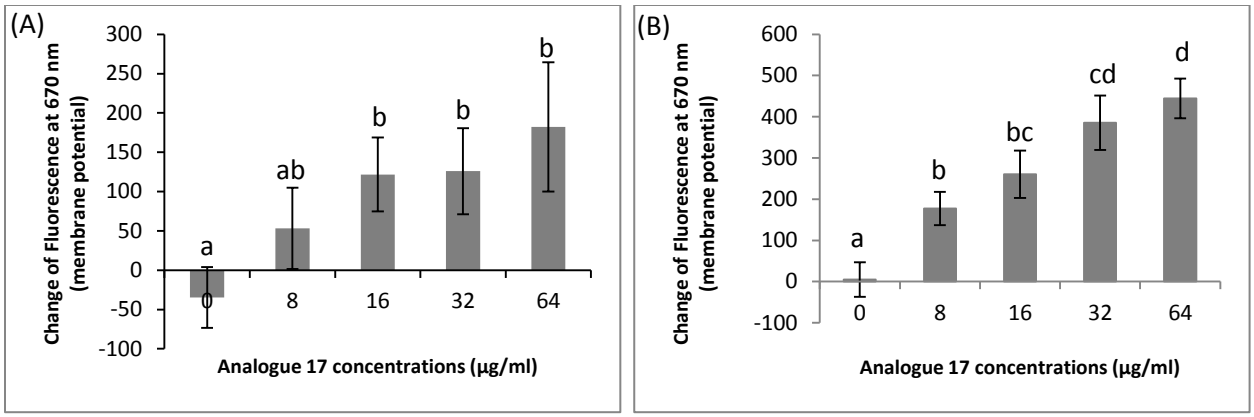

(A)
Change of Fluorescence at 670 nm (membrane potential)
300
250
200
150
100
50
0
-50
-100
a
ab
b
b
b
0
8
16
32
64
Analogue 17 concentrations (µg/ml)
(B)
Change of Fluorescence at 670 nm (membrane potential)
600
500
400
300
200
100
0
-100
a
b
bc
cd
d
0
8
16
32
64
Analogue 17 concentrations (µg/ml)

Figure 5.

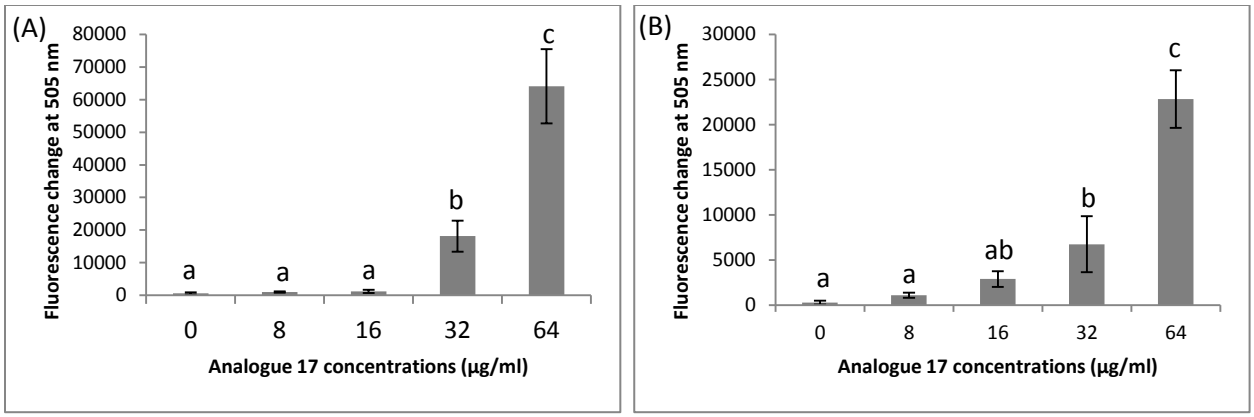
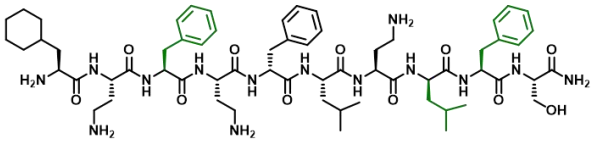

(A)
Fluorescence change at 505 nm
80000
70000
60000
50000
40000
30000
20000
10000
0
a
a
a
b
c
0
8
16
32
64
Analogue 17 concentrations (µg/ml)
(B)
Fluorescence change at 505 nm
30000
25000
20000
15000
10000
5000
0
a
a
ab
b
c
0
8
16
32
64
Analogue 17 concentrations (µg/ml)

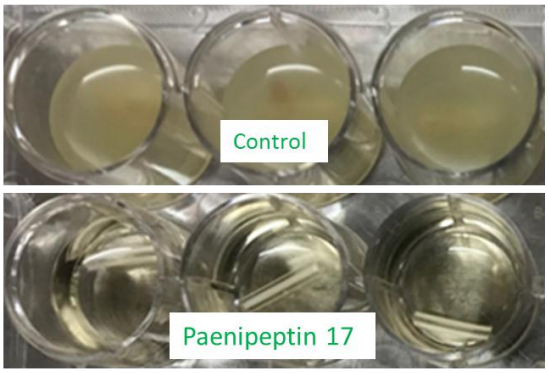
Figure 6.

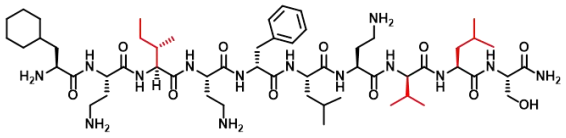
Table of Contents graphic



Paenipeptin **17**

Control *S. aureus* and *P. aeruginosa* biofilms on catheters





Paenipeptin **16**

Synergistic effect against *K. pneumoniae* and *A. baumannii*